

# ACTA ALIMENTARIA

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K. VAS

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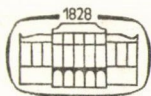
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## INVESTIGATIONS INTO THE POSSIBILITIES OF APPLICATION OF HIGH FREQUENCY ELECTRIC FIELDS TO THE BAKING OF DOUGH

T. RUNTÁG and M. DEMECZKY

(Received September 8, 1971)

The applicability of high frequency electric fields to the baking of bread was investigated on laboratory scale.

The work was centred around the determination of the electrophysical parameters: dielectric constant ( $\epsilon$ ) and loss angle ( $\delta$ ), of fundamental importance in the design of dielectric high frequency technologies.

The dependence of the chosen electrophysical parameters upon temperature was determined for a dough having the same composition as that of fine white bread in a high frequency field simulating the expected work conditions.

It was established from the results of 30 parallel experiments that the loss factor which characterizes energy uptake in the dielectric high frequency field i.e. the product of the dielectric constant and of the tangent of the loss angle, rises up to a temperature of about 75 °C after which it decreases slightly till the end of baking.

The temperature of the dough rises rapidly only in the first stage of baking, but drops significantly after vigorous evaporation has set in. The final temperature does not exceed 96 to 98 °C.

If, however, baking is performed in the dielectric high frequency field under conditions which correspond to a baking period of 4 to 4.5 minutes, energy must not be transmitted to the material before weight loss has reached one third of the desired total weight loss, or the material will lose more water than is desirable and will become dry.

The tests have shown that bread baked in the dielectric field has a looser crumb structure, its volume is by 25 to 30% greater than of bread baked by some traditional technology, while it maintains the same hedonic value.

The reproducibility of the experiments and the small deviation of the results permit of the conclusion that our values are suitable for a satisfactory estimation of prospective plant conditions.

In recent years, as a result of attempts to raise both capacity and technical level of the baking industry the Enterprise for Manufacturing and Installing Food Industry Equipment (Élelmiszeripari Gépgyártó és Szerelő Vállalat, abbreviated: ÉLGÉP) in cooperation with the Research Institute of the Baking Industry (Sütőipari Kutató Intézet, abbreviated: SIKI) has worked out a continuous technology, including the possibility of automation, for all operations of dough processing up to baking.

The problem of baking, however, remained unsolved. The thermal efficiency of the Hungarian ovens widely used in our bakeries is, according to GASZTONYI's (1968) communication, 9 to 10%, that of steam ovens 18 to 20%, but even the thermal efficiency of tunnel ovens used only in a few plants does not exceed 28 to 35%. Though the thermal efficiency of the tunnel ovens

of *Mecatherm* and *Thermoelektro*, as well as the PHS 25 and PHS 40 tunnel ovens which represent the world standard is a few per cent above 40%, their heat loss to the environment is still considerable (VODRAŽKA & HRUBY, 1970; ITSKOVICH & MARKOVICH, 1970). Primarily because of the poor thermal utilization of the ovens, their servicing is one of the most difficult operations (ÁRVAY & KULCSÁR, 1966).

Due to the poor heat conductivity of the dough it is not possible to introduce a fundamental improvement into baking technology by means of the traditional heat transfer methods.

For this very reason dielectric, high frequency heat generation, which requires no transfer medium, but acts simultaneously on the entire cross-section of the material, thereby ensuring even heating, appears to be highly promising (NUTT, 1948; SHERMAN, 1946; HOLLAND, 1963; TASNÁDI, 1966; HAFNER, 1968; WISMER, 1969).

The advantages of uniform heat transfer particularly manifest in improved quality have been treated in detail by LIISOVENKO (1965), LIISOVENKO and REKOSLAVSKIY (1968, 1969) and VOLODARSKIY (1967).

The rapid spreading of the method in the American and West European baking industry is reflected in the reports of HOLLAND (1963), MANWARING (1967) and HAFNER (1968).

We are of the opinion that by utilizing the results of Hungarian research concerned with the application of heat transfer by dielectric, high frequency heating to the food industry (DEMECKZY, 1964; GONDÁR, 1966), particularly those published in recent years (RUNTÁG & DEMECKZY, 1969), the application of the method in Hungarian bakeries has realistic possibilities.

In the research described below we tried to find out whether the application of high frequency field to bread baking is justified from the quality point of view under technical and economic conditions prevailing in Hungary.

In order to obtain results of the greatest possible practical advantage baking was performed under conditions corresponding to supposed operation conditions and the samples obtained by experimental baking were compared by means of laboratory instrumental tests to bread baked from dough prepared in the same manner and baked in the oven of the Research Institute of the Baking Industry.

By using objective test methods for assessing the quality of bread we hoped to obtain an answer to the question whether, despite the much shorter baking period, all the important changes manifest in the appearance, taste and consistency of bread had indeed taken place (GASZTONYI, 1968; AUDIDIER & BATAIL, 1968; MAKLYUKOV, 1969).

Sampling of products baked in parallel by the traditional and high frequency method, resp., was performed in accordance with the specifications of Hungarian Standard MNOSZ 6333-52, while evaluation was carried out in



conformity to Hungarian Standards MSz 20501—58 and MSz 11916—70. In addition to these Standards the methods elaborated by ROSE and SOLLE (1968), KARÁCSONYI (1970) and CSANÁD and KUN (1970) were also made use of in the evaluation of the results.

## 1. Materials and methods

### 1.1. Dough

The formula of the dough corresponded to that of which fine white bread is made:

100 kg of flour BL 80  
2 kg of yeast  
2 kg of salt  
59 kg of water

Wheat flour BL 80 for bread is, in compliance with Hungarian Standard MSz 6336—70, free of all foreign taste and odour. Its maximum ash content must not exceed 0.80% of the solids content.

In the preparation of the dough leaven was replaced by excessive amounts of yeast.

The most important parameters of wheat flour BL 80, homogenized in SIKI, were as follows:

Water uptake determined with Farinograph: 61%.

Viscosity of the flour-water emulsion determined with Amylograph at the pasting temperature of starch: 360 BU.

Gluten content: 33.5%.

Gluten spreading: 2.5 mm.

The dough to be baked by high frequency and traditional technology, resp., was homogenized for twice 20 minutes after compounding, then kneaded for 5 minutes in the laboratory kneading machine.

The dough was allowed to ferment at 30 °C for 120 minutes during which time it was twice punched back by hand.

Fermentation was performed at 30 °C, simultaneously in SIKI and the Central Food Research Institute (KÉKI).

### 1.2. Baking

The control sample was baked in the *Cimbora* type gas heated oven of SIKI. The temperature of the oven was between 240 and 260 °C and baking took 40 to 45 minutes.

For baking in dielectric field the *Brown-Boveri* type C 10 high frequency capacitive generator of KÉKI, operating at a frequency of 13.56 MHz, was used.



Table 1

*Main technical parameters of baking*

Comparative tests of samples baked by high frequency and traditional method, respectively.  
composition of

No.	Date	Baking			Baked sample		
		Time requirement	Power uptake	Thermal efficiency	Weight loss	Height	Porosity
	April, 1971	minute	kWh	%	g	mm	%
11	13	5.0	0.24	53.1	190	90	82.1
12	13	10.0	0.39	29.1	165	90	80.2
14	14	9.0	0.42	31.8	180	100	79.6
15	14	12.5	0.47	24.9	190	80	—
16	15	6.5	0.31	39.9	180	94	85.2
17	15	8.0	0.40	31.9	185	90	84.2
18	19	4.5	0.23	50.7	180	105	80.2
19	19	6.0	0.25	—	—	95	79.0
18-19C	19	45.0	—	—	—	—	76.2
20	20	4.25	0.25	48.6	180	100	82.4
21	20	4.0	0.22	47.8	180	110	88.4
20-21C	20	45.0	—	—	—	—	77.7
22	21	5.0	0.26	47.8	180	105	85.2
23	21	6.25	0.30	38.2	165	105	82.4
22-23C	21	45.0	—	—	—	—	—
22-23CT	21	45.0	—	—	—	65	79.6
24	22	4.33	0.25	45.5	190	110	85.2
25	22	6.0	—	—	—	106.5	—
24-25C	22	45.0	—	—	—	—	80.2
24-25CT	22	45.0	—	—	—	—	—
26	26	4.0	0.25	48.4	185	110	—
26-27C	26	45.0	—	—	—	—	—
26-27CT	26	45.0	—	—	—	—	—
28	27	4.5	0.27	45.0	165	110	—
29	27	8.0	0.43	25.9	165	95	—
30	19 May	6.5	0.31	31.2	190	105	—
30C	19 May	45.0	—	—	—	—	—
30CT	19 May	45.0	—	—	—	—	—

C = control sample baked by the traditional method, hand formed

CT = control sample baked by the traditional method, in a tin having the same basic area ( $345 \times 150$  mm) as that used in high frequency baking

*in the high frequency dielectric field*

The samples were baked of 1.15 kg of dough of identical quality corresponding to the fine white bread

Compressibility of crumb					
Penetrometer tests			Texturometer tests		
4	28	52	4	28	52
hours after treatment					
PU			TU		
—	—	—	—	23.8	—
—	—	—	—	22.8	—
101.5	41.0	30.1	—	—	—
—	—	—	—	—	—
113.5	—	29.6*	11.9	10.9	—
123.5	—	21.5*	—	19.6	—
132.0	60.7	43.4	6.0	13.2	22.9
121.4	55.3	38.6	10.4	23.0	—
98.8	65.7	33.5	10.4	19.8	31.6
139.2	49.9	34.3	7.2	17.6	18.8
130.3	53.5	44.5	5.8	11.7	18.9
97.0	45.5	32.1	21.8	27.4	22.7
122.5	54.7	38.6	9.1	13.3	—
114.5	61.6	36.0	7.7	14.4	28.0
—	—	—	17.2	8.6	12.1
108.5	58.4	42.0	—	—	—
131.1	78.2	49.8	4.3	13.0	—
—	—	—	—	—	—
109.1	59.2	36.1	8.9	15.7	—
149.2	71.6	65.4	—	—	—
109.5	62.5	44.3	8.2	—	—
97.2	56.2	38.8	—	—	—
88.2	46.1	42.8	10.2	—	—
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	15.7	49.7	—
—	—	—	—	—	—
—	—	—	—	—	—

\* sample tested 72 hours after baking

PU = penetrometer unit (penetration depth of the 150 g weight head into the material, in units of 0.1 mm)

TU = Texturometer unit (parameter decreasing proportionally to compressibility)



Output and changes in electric parameters of the generator were controlled by means of an outside oscillating circuit. The high frequency generator with the connected oscillating circuit is shown in Fig. 1.

The fermented dough was placed in a teflon dish between the plates of a capacitor in the external oscillating circuit in vertical parallel arrangement.

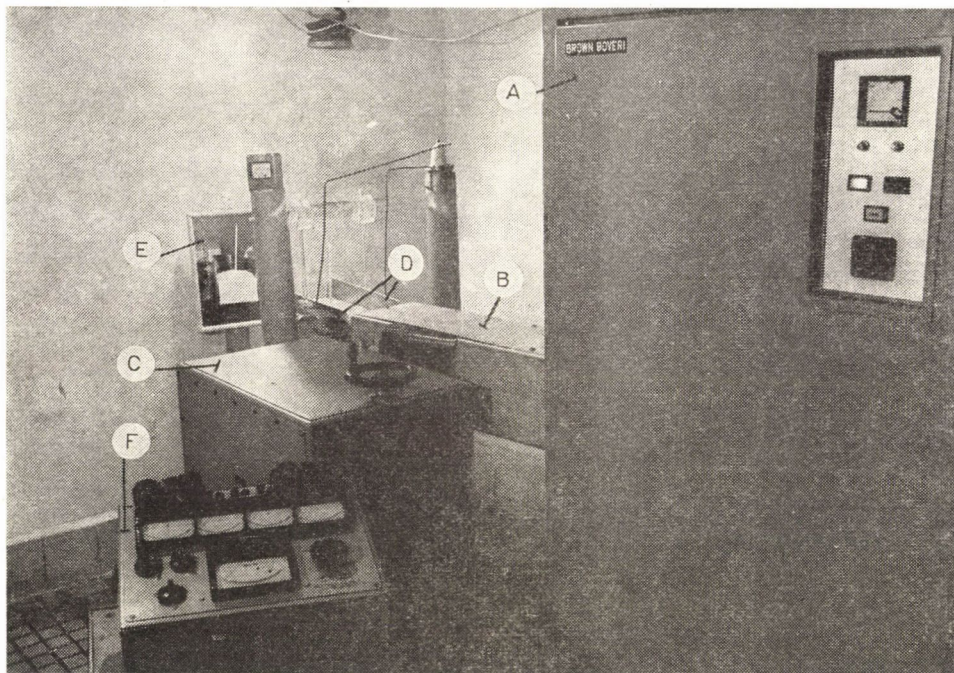


Fig. 1. Dielectric high frequency generator and its external oscillating circuit used in the experiments. A = type C 10 high frequency generator. B = coaxial cable. C = tuning inductivity. D = plates of the work capacitor. E = observation mirror. F = three phase electric outputmeter

For the continuous observation of loss in weight during baking the teflon dish with the dough was fixed on a balance.

*1.2.1. High frequency baking experiments.* The gross power requirement of baking, the output of the generator, the dimensions and relative distance, as well as the voltage between the work capacitors, the temperature and weight loss of the dough as functions of time were recorded in 30 experiments which were designed to determine the optimum baking conditions. The most important data of these records together with the results of comparing the samples are given in Table 1.

*1.2.2. Measurement of the parameters of dough baking in the high frequency field.* Changes in the temperature of dough during baking were measured with a toluene-filled, 1 °C scale thermometer.



Changes in the electrophysical properties of dough were followed with the help of a *Radelkisz* type OH 301 universal dielectrometer, system SZABÓ—B. NAGY. By accounting for the nature of the dough, the field conditions of the measuring capacitor were adjusted so that the measured values should be within the measuring limits of the instrument. This modified measuring capacitor was calibrated with the help of a substance of known dielectric constant and loss factor.

For the time of measurement the high frequency field of the generator was switched off.

The energy uptake capacity of dough was determined by reading the instrument which signals the anode performance of the generator.

Measurement of changes in volume were facilitated by the form of the rectangular base of the dish used for baking. By measuring changes in the third dimension it was possible to calculate with good approximation the expansion of the dough.

### 1.3. Comparison of breads baked by high frequency and traditional methods

Porosity characteristics of the looseness of crumb were measured in compliance with the specifications of Hungarian Standard MSz 20501—58, and the consistency of dough by the method recommended by KARÁCSONYI (1970), using a *Labor 5264* penetrometer manufactured by the Works for Laboratory Instruments (Labor Műszeripari Művek). Tests intended to give information on the hardness and cohesion properties of the material were performed by the *Texturometer* of Tominaga and Co. Ltd.

Porosity of the baked dough was measured four hours after the completion of baking, consistency was tested 4, 28 and 52 hours after baking.

## 2. Results

It appears from Fig. 2 that the effective values of the product obtained by multiplying the relative dielectric constant of the field within the work capacitors, including the dough, by the tangent of the loss angle show a tendency to increase at temperatures between 30° and 75 °C. This increase is particularly marked in the range between 30° and 50°, as well as between 65° and 75 °C. Above 75 °C there is a slight drop in the loss factor.

The energy absorption capacity of dough as a function of time changes linearly according to correlation calculations based on 60 experimental results and this indicates good reproducibility of the parallel experiments.

The temperature of dough vs. energy absorption is a hyperbolic function as illustrated by Fig. 3.

Dough can be safely loaded with 1 to 1.2 W/g power density and 5 kV high frequency voltage without the danger of breakdown. Application of lower power densities is not recommended, since in this case the expansion of dough is not satisfactory.

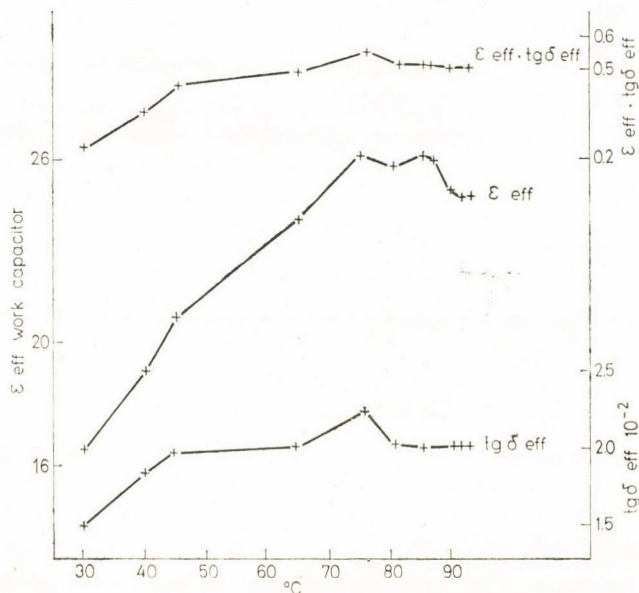


Fig. 2. The effect of dough temperature on the tangent of the loss angle ( $\text{tg } \delta$ ), the relative dielectric constant ( $\epsilon$ ) and of their product ( $\epsilon \times \text{tg } \delta$ ) in the field of the capacitor used for baking bread

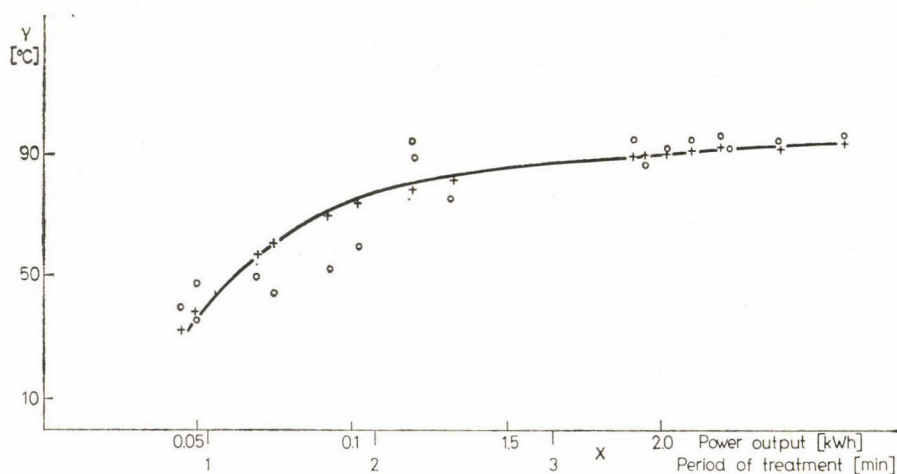


Fig. 3. Rise in the temperature of dough vs. absorbed power and duration of treatment

At a power density of 1 to 1.2 W/g the temperature of dough reaches 80 °C within 2 to 2.5 minutes, but the final baking temperature of 96 to 97 °C is attained far more slowly and the critical temperature is never surpassed.

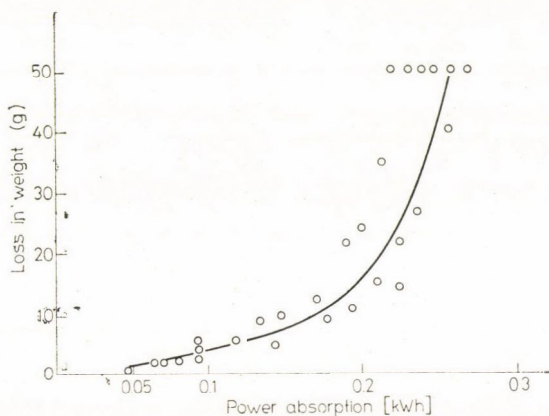


Fig. 4. Weight loss vs. power absorption

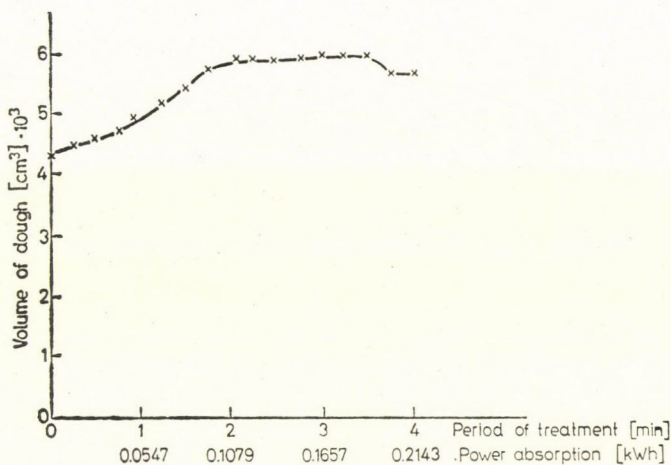


Fig. 5. Changes in the volume of dough vs. absorbed power and period of treatment

Up to 70 ° to 75 °C the moisture content of dough drops only slightly, above this temperature, however, weight loss rapidly increases and in the last phase of baking steam formation is clearly visible.

Fig. 4 shows the change in weight loss vs. energy absorption.

Changes in the volume of dough samples baked in the high frequency field as a function of energy absorption are illustrated in Fig. 5 on the basis of the evaluation of motion pictures taken during baking. Volume decrease during the last stage of baking continues to increase during cooling.



Identical negatively fixed pictures taken of the dough in the 0, 1st, 2nd and 4th minute of baking are presented in Fig. 6.

As seen in Fig. 7 out of breads of identical basic area, weight and composition, the cross section area of those baked in high frequency field is always larger than that of those baked in the conventional way.

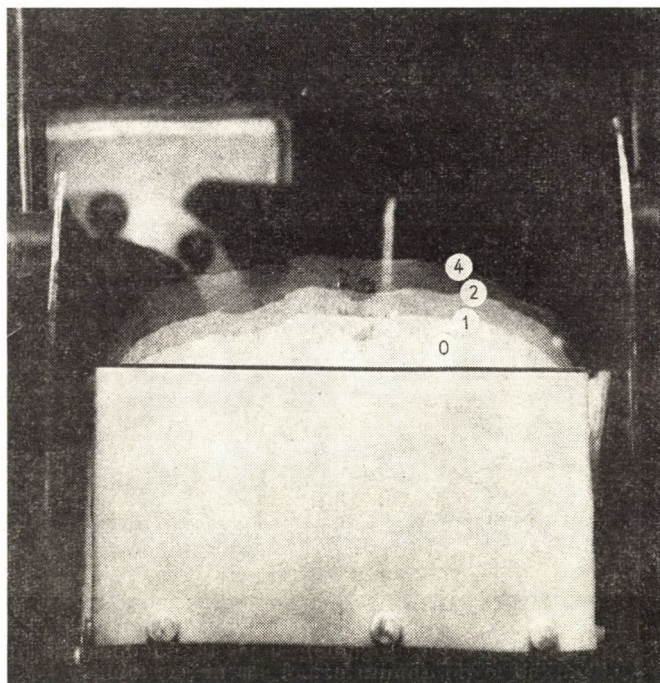


Fig. 6. Changes in the volume of dough during baking. Pictures taken on the same negative show the height of the dough, at the beginning (0), in the first minute (1), in the second minute (2), in the fourth minute (4) of baking

The lighter crumb structure of the bread prepared from the same weight of dough, but having a larger volume was confirmed, as expected, by porosity and consistency tests.

According to the data in Table 1 the porosity of the bread baked in high frequency field is 5 to 10% higher than that of the control.

The results of penetrometer values used for the further examination of crumb structure as illustrated in Fig. 8 were plotted from the data in Table 1.

The head of the penetrometer penetrates the crumb of the bread baked in high frequency field 25 to 30% deeper than that of the control. The former is inclined to preserve its lightness during ageing, as indicated by the results of measurements performed 28 and 52 hours after baking. The results of Texturometer tests were in good agreement with those obtained with the penetrometer.

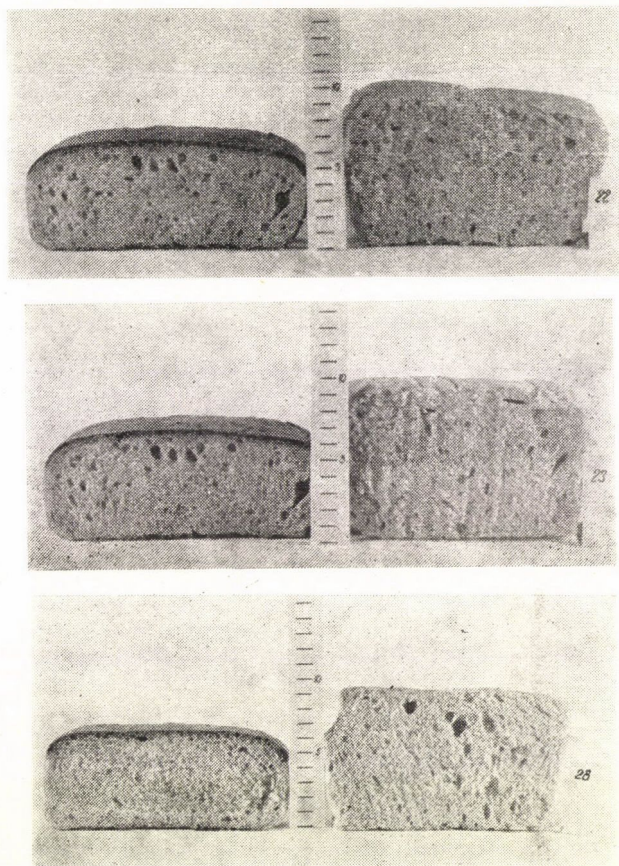


Fig. 7. Cross-section of breads. Left: bread baked by the traditional method. Right: bread baked by the high frequency method. (Experiments Nos 22, 23 and 28, Table 1.)

### 3. Conclusions

Measurement of the electrophysical parameters furnished data for the design of the most expedient arrangement for the high frequency field for baking. The other parameters necessary for developing the technology were determined from laboratory experiments simulating presumed operation conditions.

The results have confirmed the advantages pointed out in the literature; the time requirement of dielectric baking is not quite one tenth of the time requirement of traditional baking methods, and the equipment covers a considerably smaller floor space than traditional baking ovens.

It appears from the consistency tests that a baking period reduced to 4 to 4.5 minutes makes it possible to contemplate the elaboration of continuous bread production.



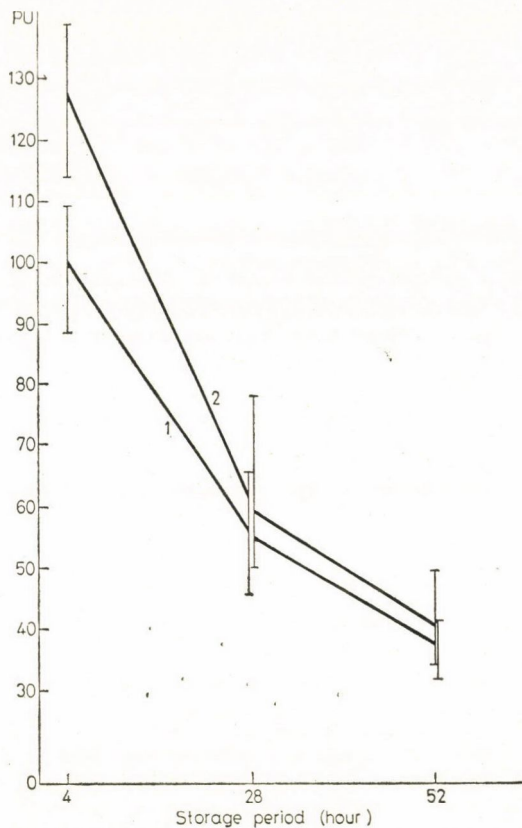


Fig. 8. Test results of the crumb properties of breads baked by the traditional method (1) and in the high frequency field (2) vs. storage period. Crumb properties measured with *Labor 5264* penetrometer and given in PU (penetrometer units)

Bread baked in dielectric field is 5 to 10% more porous than traditional bread, thus has a far lighter crumb structure and penetrometer tests indicate that even after about 12 hours storage it will possess a structure similar to that of freshly baked traditional bread. This result permits of the conclusion that bread baked in dielectric field can be stored for a longer period of time than bread available at present and consequently the consumer demand for fresh bread can be more easily satisfied. At the same time, longer storability makes the present 2-shift production, requiring a larger capacity of manufacturing machinery, superfluous, and a possibility arises of introducing continuous operation in 3 shifts. Thus in addition to the present day and night shifts it would be possible to bake also in the afternoon, or else, it might be possible to retain baking in 2 shifts without the necessity of working during the night.

Since when bread is baked in high frequency fields the entire cross-section is subjected to uniform heat effect, it will bake evenly without the

formation of a crust. If the consumer requires crust on his bread, this can be formed by infra-red lamps or by a combination with the traditional baking method. Preliminary experiments of combined baking procedures were concluded with favourable results. If no crust is required, the role of the crust as a seal against loss of moisture can be replaced by appropriate packaging.

The thermal efficiency of baking by means of dielectric heat transfer, namely the quotient of the theoretically needed heat quantity and of the heat equivalent of actually used energy is superior to the efficiency of all other baking methods, but because of the price differences between various energy carriers in Hungary the power cost of high frequency baking is higher than that of traditional baking.

According to our calculations, however, taking into account the overall effect of investment costs, amortization, power costs and wages, there is a tendency of equalization in the costs, that is, should dielectric baking methods be introduced these will not involve higher production costs.

Where power costs are not the primary factor, where the use of electric power is more easily accessible than power supplied by olefine derivatives, further where it is important to reduce the space requirement of baking, the industrial application of high frequency baking appears justified also under Hungarian conditions, as demonstrated by the results of our laboratory experiments.

\*

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## DETERMINATION OF SPECIFIC ACTIVITY OF SOME PROTEOLYTIC ENZYMES OF VEGETABLE ORIGIN

F. ÖRSI and J. MAJOR

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Enzymatic treatment appears to be the most expedient method to improve the sensory and nutritional quality of low-value meat rich in connective tissue. However, the application of the most frequently used proteolytic enzymes requires the solution of certain control problems.

When using proteolytic enzymes in the meat industry, the application of muscle tissue substrate to check the specific activity of the enzyme might provide values which can be related to the meat tenderizing effect. By modifying the semi-quantitative method of MOREAU and JANKUS (1963) we characterized the activities of proteolytic enzymes of vegetable origin (papain, ficin and bromelain), on muscle tissue as a substrate at optimum temperature, by the quantity of the liberated peptide nitrogen soluble in 50% alcohol and determination by means of the biuret reagent; and this was referred to 1 g of dry enzyme protein.

To demonstrate the applicability of the method the entire temperature: specific activity curve of three commercial enzyme preparations and the pH—specific activity curve of the enzyme preparation ficin were determined.

Activity determined in this manner is, in a wide concentration range, proportional to the quantity of enzyme and there is an unequivocal relationship between specific activity and the "meat tenderizing" ability of enzymes.

In recent years, demand for meats containing proteins of complete biological value has increased all over the world. At the same time it has become desirable to utilize in the meat industry proteolytic enzymes to improve meats of poorer quality which are less valuable from the aspect of the consumer (hard to digest, tough meat).

When introduced into meat these enzymes will alter, by way of their proteolytic action, the structure of the connective tissue and will thus exert a favourable influence on the tenderness of prepared meat (TELEGDY KOVÁTS & SZILAS-KELEMEN, 1965).

For the general use of proteolytic enzymes in the Hungarian meat industry first some problems of application and control have to be solved, including the dosage of enzymes which again demands an easily applicable method of activity determination\* (ÖRSI, 1971).

These experiments were aimed at developing a method for the determination of activity which

\* By activity the activity of 1 g of enzyme protein, thus the specific activity is understood.

a) determines enzyme activity on muscle tissue substrate, since this is the only way to ensure evaluation of the results in relationship to the "meat tenderizing" effect, and

b) considering the temperature optimum of enzymes and the conditions under which meat is processed, determines activity at 60 °C. As a matter of fact, this method is suitable for plotting the entire activity-temperature curve.

When developing our method for the determination of activity, we started from the procedure suggested by MOREAU and JANKUS (1963) which had originally been intended for the semi-quantitative determination of the amount of papain enzyme used in the treatment of beef. This method characterizes the amount of enzyme by measuring the quantity of alcohol soluble peptides liberated from minced muscle tissue and utilizes the exopeptidase nature of vegetable proteolytic enzymes which is related to the meat tenderizing effect.

This semi-quantitative method was made quantitative by relating the liberated peptide nitrogen to the quantity of the applied biuret reagent and by photometric evaluation.

## 1. Materials and methods

### 1.1. Enzyme preparations

In the experiments three commercial enzyme preparations, papain, ficin and bromelain were used. All three are manufactured by Nutritional Biochemicals Corporation, Cleveland, Ohio.

### 1.2. Substrate

Muscle tissue was chosen as the substrate. About 250 g of beef freed of fat and tendons (longissimus dorsi) were passed three times through the mincer using a disc with holes of 1.5 mm diameter; the minced meat was thoroughly homogenized, and kept up to the time of the test in a closed container in the refrigerator at temperatures between 0° and 5 °C for a period not longer than 2 days.

### 1.3. Derivatographic determination of the moisture content of enzyme preparations

The moisture content of enzyme preparations was determined in the Paulik-Paulik-Erdey *Derivatograph* (manufactured by the Hungarian Optical Works, MOM). 200 to 400 mg of the enzyme preparation were dried by raising the temperature from room temperature to 300 °C at a rate of 5 °C/minute and changes in weight as well as the differential thermal analysis (DTA) curve,



expressing enthalpy changes during the process, were recorded. The weight loss taking place in the endothermic process up to 200 °C was accepted as the moisture content for all three preparations.

#### *1.4. Determination of the proteolytic activity of enzyme preparations*

*1.4.1. Incubation and removal of proteins.* Activity was determined in an ultrathermostat at  $60\text{ °C} \pm 0.05\text{ °C}$ , 5.0 g of the muscle tissue substrate was mixed in a test tube of 20 mm diameter, using a glass rod with 14 ml of distilled water and preheated in the thermostat to the test temperature. When the homogenate had reached the temperature of the thermostat 6.0 ml of the enzyme solution which had carefully been heated to the test temperature were added to the substrate and the mixture incubated for 30 minutes. The sample was then filtered while still warm. Filtration through a 9 cm diameter Macherey, Nagel and Co. filter paper took 15 minutes and the filtrate was collected in a test tube with 0.1 ml rulings. After cooling to room temperature the volume of the filtrate was accurately read and the same volume of 96% ethanol added. The mixture was allowed to stand for 10 minutes and the precipitated protein could be easily filtered through a 9 cm diameter Macherey, Nagel and Co. filter paper.

*1.4.2. Determination of peptide nitrogen.* The filtrate free of protein was diluted with distilled water (to an extent depending upon the quantity of peptides) in known proportion, mostly to 5 to 10 times the initial volume. To 2.0 ml of the diluted solution, 1 ml of 96% ethanol and 1 ml of biuret reagent with 0.02 mole copper content per litre were added. Optical density of the solution was read 30 minutes after mixing by means of the *Spektromom 360* photometer (manufactured by MOM) at 560 nm against a blank prepared under identical conditions.

*1.4.3. Plotting of the calibration curve for the determination of peptide nitrogen by means of the biuret method.* 100 g of muscle tissue were mixed with 500 ml of distilled water and 8 g of ficin and incubated at 60 °C for 1, 2 and 3 hours, resp., filtered while still warm, the filtrate cooled to room temperature and mixed with an equal volume of 96% ethanol. After 10 minutes of standing the protein precipitate was filtered off and the nitrogen content of the filtrate determined by the Kjeldahl method.

From other aliquots of the filtrate various dilutions were prepared and their optical density after the reaction with biuret reagent was read according to para. 1.4.2.

#### *1.5. Determination of the effect of temperature on the activity of the enzymes*

The effect of temperature on the activity of enzyme preparations was studied in the temperature range between 30° and 100 °C in the ultrathermo-



stat as described in para. 1.4. For temperatures of  $+8^{\circ}$  and  $+5^{\circ}\text{C}$  the samples were cooled to, and maintained at, the desired temperature in refrigerators. Temperatures of  $-7^{\circ}$  and  $-20^{\circ}\text{C}$  were ensured by appropriate refrigerating machines. The incubation period was extended to 24 hours and the processing of the sample was continued in accordance with para. 1.4.

#### *1.6. Determination of the effect of pH on enzyme activity*

The effect of pH on ficin was investigated at  $60^{\circ}\text{C}$ . Determination was performed as described in para. 1.4, but the substrate was mixed with 14 ml of a 0.2 M Sörensen phosphate buffer of various pH values. The actual pH values were determined at the end of the incubation period in the filtrate prior to the addition of alcohol. The pH values were checked by a precision pH-meter (type OP-205, manufactured by *Radelkisz*) and a combined glass electrode.

#### *1.7. Enzymatic treatment and preparation of meat slices*

Enzymatic treatment was performed according to the specifications worked out, under contract from the Central Food Research Institute (KÉKI), by the Department of Food Chemistry of the Technical University, Budapest (1968): slices of about 1 cm thickness and 100 g weight of longissimus dorsi were pricked on both sides with a pin-brush (100 pins per  $100\text{ cm}^2$ ) and sprayed with the specified quantity of the enzyme preparation divided equally on both sides. As soon as the enzyme preparation became wetted the slices were prepared by stewing in an edible oil – water mixture, under cover, for 90 minutes.

#### *1.8. Sensory judgement of the tenderness of cooked meat slices*

The tenderness of the cooked meat was determined by a panel of six trained persons. Tenderness of the slices was characterized by the number of chewings necessary for swallowing a 15 mm diameter, 1 cm thick disc of the prepared meat at room temperature.

#### *1.9. Instrumental determination of the tenderness of cooked meat slices*

The tenderness of cooked meat slices was determined in the modified *Höppler Cconsistometer* using the method developed in the Department of Food Chemistry of the Technical University, Budapest (1968) and was characterized by the force necessary to cut a 1-cm thick meat slice with a knife of 1 cm length.

## 2. Results

### 2.1. Determination of some important characteristics of the applied enzyme preparations

The moisture content of the enzyme preparations was determined by the derivatographic method described in para. 1.3, their protein content was determined by the Kjeldahl method and the results were summed up in Table 1. The protein content was calculated from the experimentally obtained nitrogen content by using a multiplication factor of 6.25 and the result related to the solids content of the preparation.

Table 1

*Some important characteristics of the enzyme preparations used in the tests*

Enzyme preparation	Identification number	Moisture content %	Protein content % solids
Papain	5360	10.2	98.0
Ficin	6380	12.2	96.0
Bromelain	6499	14.6	98.5

### 2.2. Results of the determination of alcohol soluble peptide nitrogen

The reaction product of the filtrate, which has been deproteinized with alcohol, and of the biuret reagent shows maximum absorption at 520 nm as illustrated by the absorption curve in Fig. 1.

The amount of peptide nitrogen was determined from the optical density measured with the spectrophotometer using the calibration curve plotted in accordance with para. 1.4.3. The points of the curve shown in Fig. 2 lie along a straight line which can be characterized by the correlation coefficient  $r = 0.998$ . The equation of the straight line is given in the Figure, its axial section, according to calculations, does not significantly deviate from zero.

There is no essential difference between the curves obtained for various incubation periods; the points corresponding to a 3-hour incubation period lie, however, below the straight regression line which has been calculated from all the points.

### 2.3. Results of activity tests

Fig. 3 shows the amount of alcohol soluble peptide nitrogen liberated by the enzyme as a function of time and of enzyme concentration.



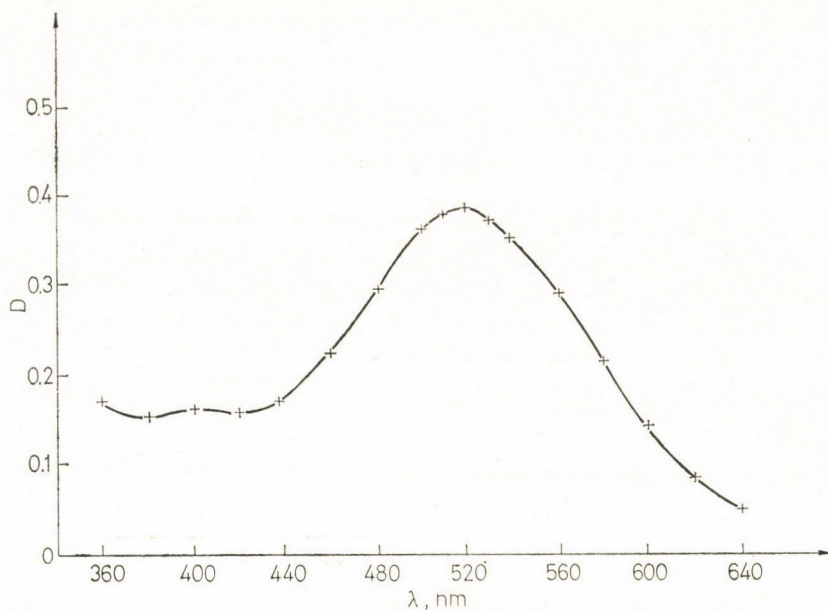


Fig. 1. Optical density ( $D$ ) of the reaction product of the deproteinized filtrate of beef muscle tissue suspension with biuret reagent as a function of wavelength (degradation with ficin, removal of protein by addition of alcohol to the degraded suspension)

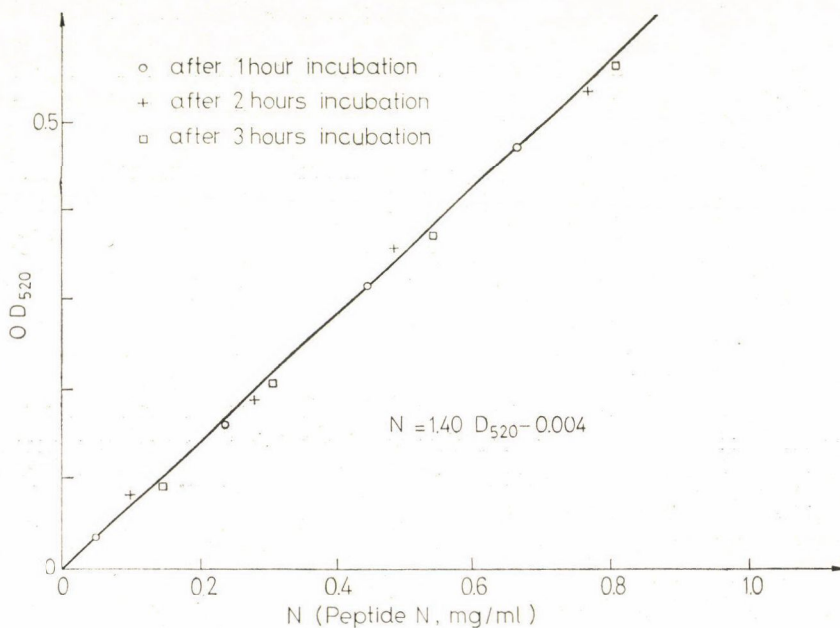


Fig. 2. Calibration curve for the determination of nitrogen content by means of the biuret method. Temperature: 60 °C; enzyme: ficin



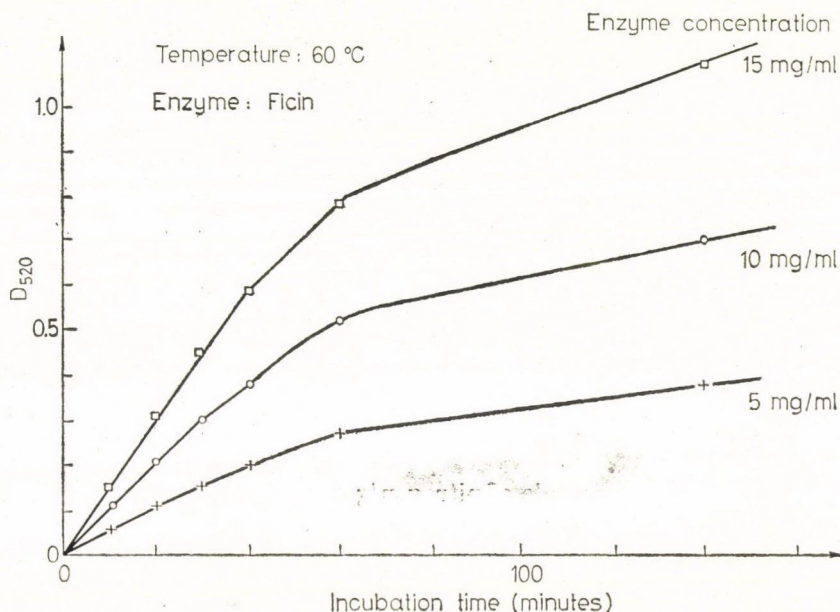


Fig. 3. Quantity of alcohol soluble peptides liberated from samples treated with various quantities of ficin and incubated at 60 °C, expressed in terms of the optical density of the reaction product with biuret

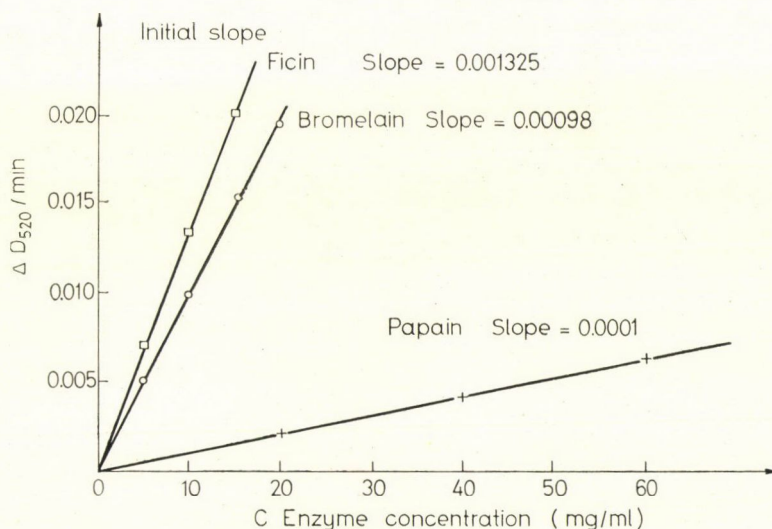


Fig. 4. Correlation between the slope of the initial (30-minute) section of the optical density vs. incubation period curves and enzyme concentration

The first straight section takes 40 to 60 minutes after which the rate of degradation drops. The slope of the section pertaining to the first 30 minutes is plotted vs. the enzyme concentration for the three preparations under investigation in Fig. 4.

There is a linear correlation between the quantity of enzyme and the slope as calculated from the initial 30-minute periods of the curves.

In the investigated concentration range, the coefficient of correlation,  $r = 0.98$ .

On the basis of what has been said before, (specific) activity was characterized by the amount (micromoles) of nitrogen — soluble in 50% alcohol and determined by the biuret method — which had been liberated under the described conditions by 1 g of enzyme protein in 1 minute:

$$PA = \frac{67 \cdot D \cdot V}{G}$$

where

$PA$  = the activity; micromole of nitrogen/g of enzyme protein · minute

$D$  = optical density measured, after the addition of the biuret reagent, at 520 nm

$V$  = volume of filtrate, ml

$G$  = solids content of enzyme protein, g.

The multiplication factor 67 arises from the conversion of optical density values into N, taking into consideration the dilutions.

The activity values of the investigated enzyme preparations at 60 °C are summarized in Table 2.

Table 2

*Specific activity of the enzyme preparations at 60 °C*

Enzyme preparation	PA micromole N/min · g enzyme protein
Papain	200
Ficin	2430
Bromelain	1970

#### *2.4. Accuracy of the method used for the determination of activity*

Accuracy of the activity values determined by the suggested method is a function of several factors. The results of altogether 50 determinations carried out on substrates originating partly from the same and partly from different animals and with various enzyme quantities were evaluated by means of analysis of variance to determine the size of the main components of error. The results of this analysis are summarized in Table 3.

### 2.5. The dependence of activity on temperature

The temperature dependence of enzyme activity is a very important factor in the estimation of the meat-tenderizing effect of enzymes. Proteolytic activity responsible for the tenderizing effect proceeds under greatly varying temperature conditions in the course of the preparation of meat.

The temperature dependence of activity is illustrated in Fig. 5 in a very wide temperature range between  $-20^{\circ}\text{C}$  and  $+100^{\circ}\text{C}$ .

At extremely low temperatures the method had to be modified to some extent by increasing the period of incubation.

In a semi-logarithmic plot (Fig. 5) the section between  $-20^{\circ}\text{C}$  and  $+60^{\circ}\text{C}$  of the activity vs. temperature curve is a straight line and parallel lines correspond to the three enzyme preparations. The equation of these sections was determined by means of the method of least squares, and the following equations were obtained:

$$\text{Papain} \quad \log PA = 0.0481 t + 0.314$$

$$\text{Ficin} \quad \log PA = 0.0481 t + 1.190$$

$$\text{Bromelain} \quad \log PA = 0.0478 t + 0.916$$

where

$PA$  = proteolytic activity and

$t$  = temperature in  $^{\circ}\text{C}$ .

The slopes of the lines indicate that an increase of  $10^{\circ}\text{C}$  raises enzyme activity by a factor of three in the temperature range between  $-20^{\circ}\text{C}$  and  $+60^{\circ}\text{C}$ .

### 2.6. Effect of pH on enzyme activity

Since the muscle tissue substrate used in the activity determinations is of a neutral pH, it changes in the mildly acidic range which may influence the determination of activity. In order to be able to estimate this effect, the pH dependence of activity was determined by the method described in para. 1.6. Figure 6 shows the effect of pH on the activity of ficin.

Table 3

*Influence of the main sources of error on the accuracy of activity determination*

	Optical density range	Coefficient of variation of the activity value, %
Identical meat sample	0.3 — 0.7	6 — 8
Varying meat sample	0.05 — 0.02	11 — 15
	0.3 — 0.7	9 — 11



In agreement with data found in the literature the enzyme activity of ficin is almost independent of pH in neutral and weakly acidic media, thus it was not necessary to use a special buffer solution when measuring activity.

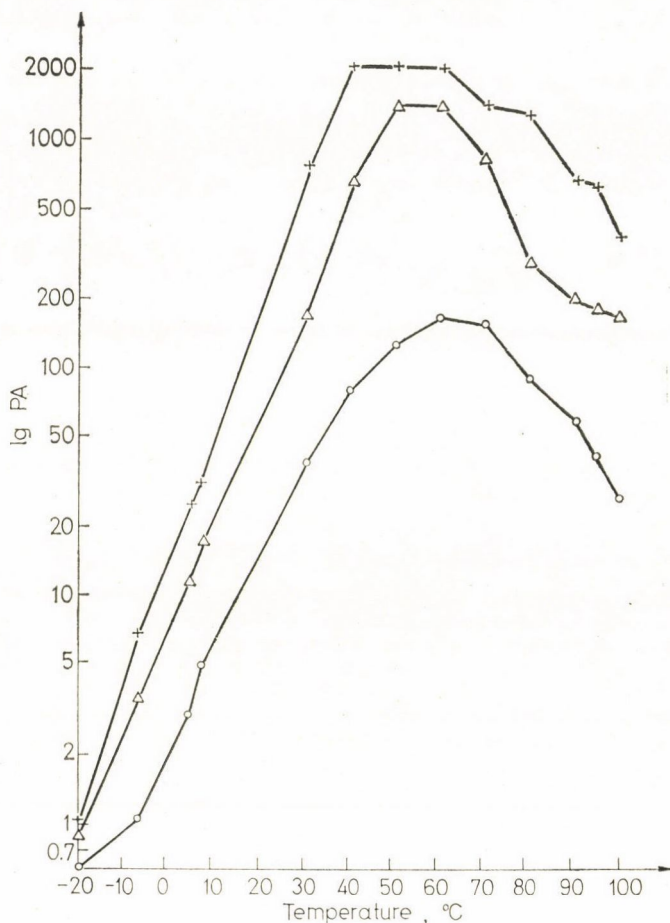


Fig. 5. Temperature dependence of the activity of enzyme preparations.  
+—+ ficin;  $\Delta$ — $\Delta$  bromelain; O—O papain

### 2.7. Comparison of the meat tenderizing effect of enzyme preparations at identical activity values

In order to compare actual meat tenderizing effects, experiments were carried out with amounts of the three enzyme preparations having identical activities.

Meat slices were treated with amounts of the enzymes which had the same activity (200 PA/100 g), using the method described in para. 1.7 and

the tenderness of stewed meat slices was judged by sensory tests and instrumental measurements as described in paras 1.8 and 1.9. The results are summed up in Table 4.

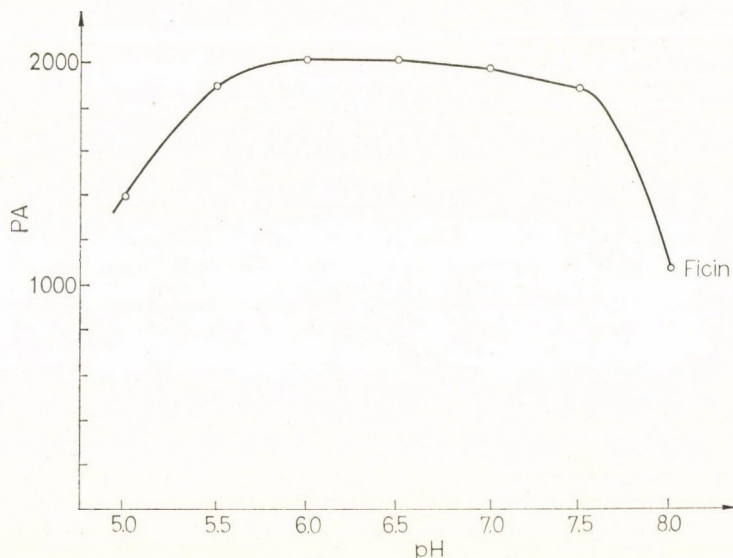


Fig. 6. pH dependence of ficin activity

Accuracy of sensory scores (confidence interval pertaining to 95% probability level):  $\pm 3$ , accuracy of the determination of the force needed for cutting:  $\pm 500$  g.

Both sensory and instrumental tests indicated that, compared to the controls, the meat samples treated with various enzyme quantities having identical activities are more tender. Furthermore, no significant difference could be established between the various enzyme preparations at the same activity levels.

Table 4

*Estimation of the tenderness of meat slices which had been treated with enzyme doses of 200 PA activity units and prepared by stewing*

Treatment	Quantity of enzyme preparation taken	Sensory score	Force needed for cutting, g · cm
Untreated	—	16	6000
Papain	1.00 g	9	3500
Ficin	0.08 g	10	4000
Bromelain	0.10 g	11	4000



### 3. Conclusions

It appears from the results that our new method for measuring activity which we developed from the semi-quantitative method of MOREAU and JANKUS (1963) is suitable for the quantitative determination of enzyme activity.

When measuring activity it is not necessary to apply a special buffer solution to ensure a constant pH, for in the neutral or mildly acid pH range, ensured by the buffer capacity of the muscle tissue used as substrate, there is no significant change in activity.

The method is applicable to the determination of the temperature dependence of enzyme activity in a wide temperature range, as shown for three commercial enzyme preparations in the temperature range between  $-20^{\circ}\text{C}$  and  $+100^{\circ}\text{C}$ .

The activity determined by the above method is in good relationship with the meat tenderizing effect of enzymes, since both sensory and instrumental tests failed to reveal any significant difference in the meat tenderizing effect of different enzyme preparations used at identical activity levels as determined by the new method.

This indicates that our method is suitable for the determination of enzyme activity of proteolytic enzymes of vegetable origin used in the meat industry.

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## METHOD FOR THE DETERMINATION OF LIPASE ACTIVITY AND ITS APPLICATION TO KINETIC INVESTIGATIONS

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A simple method, suitable for the serial investigation of lipase activity, was developed by the modification of YAMADA's (1962) method. This modification improves the sensitivity of the method and the obtained linear correlations are valid for a well measurable period. Olive oil substrate was used from which a stable emulsion was prepared by the addition of lecithin.

The applicability of the method was tested by kinetic experiments on model enzymes. A linear correlation was obtained between reaction time and the quantity of the end product, as well as between the initial quantity of enzyme and the amount (in  $\mu$ mole) of liberated fatty acid for a well measurable period. There is no substrate inhibition within this linear section, as evidenced by the validity of SELWYN's correlation.

For the investigated NBC lipase preparation (Nutrition Biochemicals Corp., Cleveland, Ohio) the following data were obtained: value of the Michaelis constant:  $4.86 \cdot 10^{-2}$  mole/litre, maximum reaction rate at 0.25 mg/ml enzyme concentration:  $1.01 \cdot 10^{-4}$  mole/litre  $\cdot$  minute, and at 0.5 mg/ml enzyme concentration:  $1.94 \cdot 10^{-4}$  mole/litre  $\cdot$  minute.

Lipase hydrolyzes the esters of long-chain fatty acids and polyvalent alcohols. Several methods are described in the literature for the determination of lipase activity; the majority of these is based on the titration of fatty acids liberated in the course of hydrolysis. The most frequently used substrates are: tributyrin, Tween, trioctanin and olive oil. Since these esters are water insoluble, they require the addition of some emulsifier, which might be sodium taurocholate (SOMKUTI *et al.*, 1969), polyvinyl alcohol (YAMADA *et al.*, 1962) or gum arabic (DESNUELLE, *et al.*, 1959).

The preparation of a stable emulsion is one of the most important requirements in the determination of lipase activity, because enzyme activity is influenced not only by substrate concentration, but also by the size of the surface in contact with the enzyme solution. A breakdown of the emulsion during the measurement of activity will lead to an uncontrollable drop in reaction rate.

In this paper we wish to report on the method which we have developed for the measurement of lipase activity using lecithin as emulsifier of the olive oil substrate. By means of the stable emulsion obtained in this manner we determined the enzyme concentration, Michaelis constant and maximum reaction rate of a model lipase preparation for which we used the product of Nutrition Biochemicals Corp., Cleveland, Ohio (hereinafter: NBC).



### 1. Materials and methods

The object of our work was to elaborate a simple method of lipase activity determination suitable for serial tests. We used olive oil as substrate (L'union centrale des cooperatives oleicoles, Zitouna, Tunis) and sodium taurocholate (Reanal, Hungary) or polyvinyl alcohol (PVA: Koch-Light Laboratories Ltd., Great Britain) were tried as emulsifiers. The emulsion containing the oil, emulsifier and buffer had, in the first case, begun to separate already when pipetted, and in the second case during incubation. Conditions were somewhat more favourable when the enzyme — substrate mixture was agitated on the shaking machine during the period of the reaction, but, when reaction was more protracted, separation occurred even under these conditions. We have therefore applied lecithin, commonly used in the vegetable oil industry, as emulsifier.

Lecithin was obtained from the Vegetable Oil Works, Rákospalota, Hungary, and was first added as stabilizer in quantities of 0.5% to the substrate compounded in accordance with YAMADA's (1962) method, but later it was found that in the presence of lecithin the application of PVA is superfluous. The composition of the YAMADA substrate mixture is as follows: 1 : 3 mixture of olive oil and 2% PVA solution agitated under cooling in a Biomix homogenizer for 10 minutes.

We determined lipase activity in the following manner: a mixture of  
5 ml of substrate (containing 1.25 ml of oil)  
4 ml of buffer  
1 ml of enzyme solution

was shaken in a 100 ml conical flask in a 40 °C thermostat at a rate of 140 to 160 strokes per minute. When reaction time was over, the reaction was stopped by the addition of 20 ml of a 1 : 1 mixture of acetone-ethanol, 3 drops of phenolphthalein and 10 ml (or as many times 10 ml as necessary for keeping the mixture alkaline) of 0.05 *N* NaOH, and excess alkali was titrated with 0.02 *N* HCl. A control was prepared in a similar manner; it differed from the sample solely by containing a boiled enzyme solution. The quantity of oleic acid liberated in course of the hydrolysis process was calculated from the difference between the titration values of the control and of the sample.

Activity is given in international units. The enzyme quantity liberating from the reaction mixture 1  $\mu$ mole of fatty acid per minute, was considered to have unit activity. The enzyme concentration of the preparation is given by the number of  $\mu$ moles of fatty acid liberated by 1 g of enzyme during one minute. The enzyme concentration of 1 g of the preparation was calculated from the following formula:

$$\text{Activity/g} = \frac{(V-M) \cdot f \cdot 1000}{0.05 \cdot x \cdot t}$$

where

- $V - M$  = difference between the titration values of the control and of the sample containing active enzyme, ml;  
 $f$  = factor of the 0.02  $N$  HCl solution;  
 $x$  = initial weight of the enzyme, mg;  
 $t$  = reaction time, minute;  
0.05 = difference in the quantity of titrating solution due to the liberation of 1  $\mu$ mole of fatty acid.

The reaction mixture should not contain more than 0.3–3.5 units of enzyme.

Since lipase preparations of different origin have different optimum values of pH, the pH of the buffer will depend on the enzyme under investigation. We found that under our conditions 0.1  $N$  phosphate buffer (pH 8.0) is suitable.

The coefficient of variation of the method is 8.6%.

## 2. Results

Applying the above method to the measurement of activity, three enzyme concentrations (0.25, 0.5 and 1.0 mg/ml) were prepared of the NBC lipase preparation used as model enzyme and the relationship between reaction time and the quantity of liberated fatty acid in  $\mu$ mole was plotted (Fig. 1).

It appears from Fig. 1 that in the initial phase there is a linear relationship between the quantity of fatty acid liberated during the hydrolysis of olive oil and the reaction time. Depending on the initial quantity of the enzyme the duration of this linear section is 1 to 2 hours. This linear section lends itself to the calculation of enzyme activity and to the comparison of the enzyme concentrations of various preparations.

Fig. 2 shows the correlation between enzyme concentration and  $\mu$ moles of liberated fatty acid for three reaction times (15, 30 and 60 minutes). It appears from Fig. 2 that in all three cases this correlation, too, is linear.

Fig. 3 shows for all three enzyme concentrations the absence of inactivation during the incubation of the enzyme under the applied experimental conditions. According to SELWYN's (1965) communication, if quantities of the reaction products formed are plotted vs. the product of enzyme concentration and reaction time, the values for various concentrations will lie on a straight line in the absence of inhibition.

From the equation of the joint regression line of the values obtained for three different enzyme concentrations, the enzyme concentration of the NBC lipase preparation was found to be 342 IU/g.



The relationship between enzyme activity and substrate concentration is illustrated in Fig. 4. In the case of heterogeneous systems, such as an oil — water emulsion, a normal Michaelis correlation will be obtained only if there is an excess substrate surface with reference to the enzyme, since reaction rate is influenced not only by the substrate concentration, but also by the accessible surface.

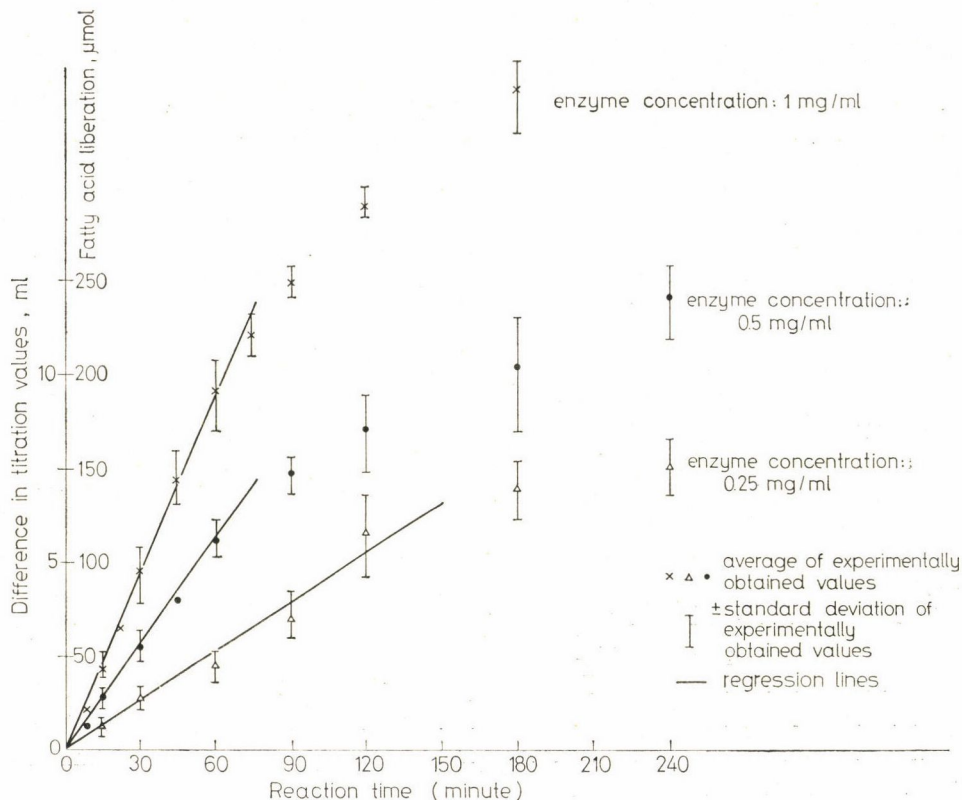


Fig. 1. Activity of the NBC lipase preparation vs. reaction time. pH 8.0,  $t = 40^{\circ}\text{C}$ , substrate: 5 ml of emulsion, enzyme concentrations: 0.25, 0.5 and 1.0 mg/ml, resp. Equations of regression lines, where  $x$  is the reaction time, minute,  $y$  the quantity of liberated fatty acid,  $\mu\text{mole}$ : for an enzyme concentration of 0.25 mg/ml:  $y = -2.40 + 0.896x$ ,  $r = 0.985$ ; for an enzyme concentration of 0.5 mg/ml:  $y = 6.8 + 1.78x$ ,  $r = 0.995$ ; for an enzyme concentration of 1.0 mg/ml:  $y = 13.6 + 2.94x$ ,  $r = 0.990$

In Fig. 5 the reciprocal presentation of the correlation was plotted according to Lineweaver and Burk. In case of both enzyme concentrations the experimentally obtained points lie along a straight line. The value of the Michaelis constant and the maximum rate of the enzyme reaction were calculated from the equations of the regression lines connecting the points. According to the method developed by us, enzyme activity is determined in the

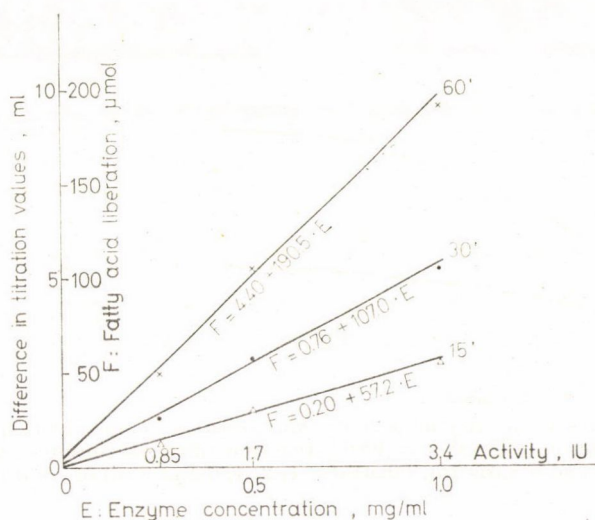


Fig. 2. Activity of the NBC lipase preparation vs. enzyme concentration. pH 8.0,  $t = 40^\circ\text{C}$ , substrate: 5 ml of emulsion, enzyme concentrations: 0.25, 0.5 and 1.0 mg/ml, resp., reaction times: 15, 30 and 60 minutes. Equations of regression lines where  $x$  is the enzyme concentration in mg/ml,  $y$  the quantity of reaction products in  $\mu\text{mole}$ : 15 minutes:  $y = 0.20 + 57.2 x$ ,  $r = 0.9928$ ; 30 minutes:  $y = 0.76 + 107.0 x$ ,  $r = 0.9975$ ; 60 minutes:  $y = 4.40 + 190.5 x$ ,  $r = 0.9962$

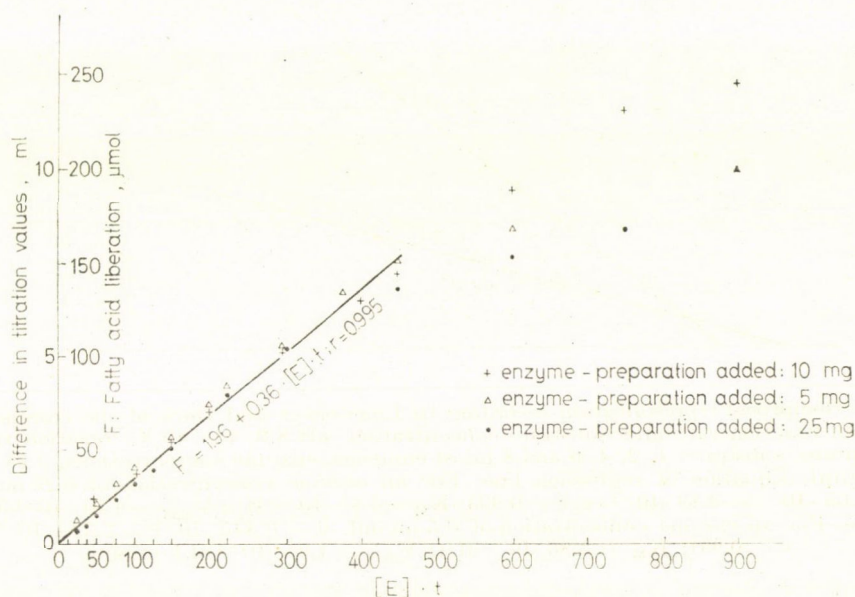


Fig. 3. SELWYN's correlation for enzyme activity vs. the product of initial enzyme content and reaction time. pH 8.0,  $t = 40^\circ\text{C}$ , substrate: 5 ml of emulsion, enzyme concentrations: 0.25, 0.5 and 1.0 mg/ml, resp., reaction times: 5, 10, 15, 30, 45, 60, 75, 90, 120, 180, and 240 minutes. Equation of the common regression line:  $y = 1.96 + 0.34 x$ ,  $r = 0.9952$



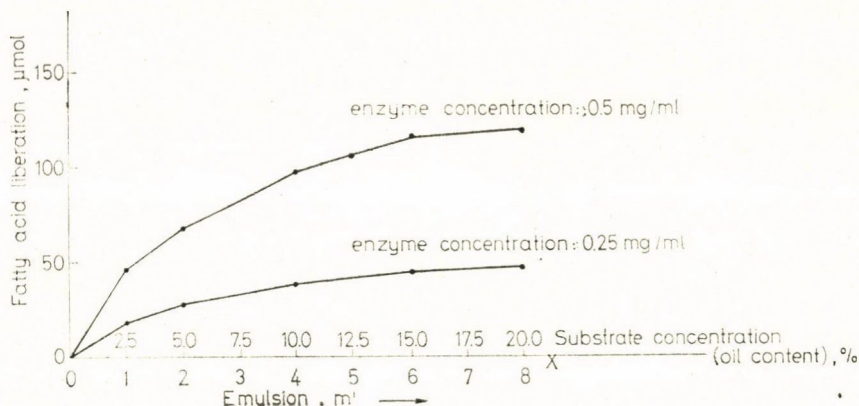


Fig. 4. Correlation between enzyme activity and substrate concentration in the case of NBC lipase preparation. pH 8.0,  $t = 40^\circ\text{C}$ , reaction time: 60 minutes, enzyme concentrations: 0.25 and 0.5 mg/ml, substrate: 1, 2, 4, 6 and 8 ml of emulsion, resp.

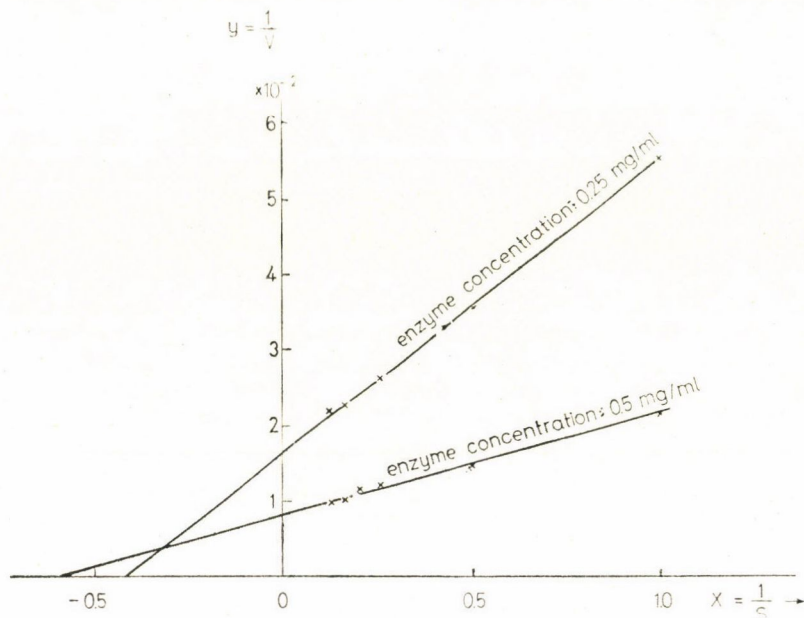


Fig. 5. Reciprocal representation according to Lineweaver and Burk of the correlation between reaction rate and substrate concentration. pH 8.0,  $t = 40^\circ\text{C}$ , reaction time: 60 minutes, substrate: 1, 2, 4, 6 and 8 ml of emulsion, enzyme concentrations: 0.25 and 0.5 mg/ml. Equation of regression line: For an enzyme concentration of 0.25 mg/ml  $y = 1.65 \cdot 10^{-2} + 3.89 \cdot 10^{-2} \cdot x$ ,  $r = 0.993$ ;  $K_M = 5.87 \cdot 10^{-2} \text{ M/l}$ ;  $V_{\max} = 1.01 \cdot 10^{-4} \text{ M/l} \cdot \text{minute}$ . For an enzyme concentration of 0.5 mg/ml:  $y = 0.845 \cdot 10^{-2} + 1.329 \cdot 10^{-2} \cdot x$ ,  $r = 0.991$ ;  $K_M = 3.85 \cdot 10^{-2} \text{ M/l}$ ;  $V_{\max} = 1.97 \cdot 10^{-4} \text{ M/l} \cdot \text{minute}$

presence of 5 ml of emulsion, corresponding to 0.125 mole/litre of substrate concentration. The enzyme acts at half of its maximum reaction rate in the presence of 1.96 ml of emulsion, thus the value of the Michaelis constant is  $4.86 \cdot 10^{-2}$  mole/litre.

For an enzyme concentration of 0.25 mg/ml the maximum reaction rate was  $1.01 \cdot 10^{-4}$  mole/litre  $\cdot$  minute, at an enzyme concentration of 0.5 mg/ml this rate was  $1.97 \cdot 10^{-4}$  mole/litre  $\cdot$  minute.

### 3. Conclusions

A simple method was developed for the determination of the activity of lipase preparations. In this method which is also suitable for serial tests, the substrate was olive oil to which 2% of lecithin was added as emulsifier. In this way an emulsion was obtained which was more stable than the one prepared with polyvinyl alcohol or sodium taurocholate, as recommended in the literature. The enzyme content of the reaction mixture may vary between 0.3 and 3.5 IU. Activity was measured at 40 °C and pH 8.0. The coefficient of variation of the method is  $\pm 8.6$ .

In a well defined phase of the hydrolysis of olive oil a linear correlation was obtained between reaction time and the quantity of decomposed substrate (Fig. 1).

The correlation between enzyme concentration and the quantity of liberated fatty acid is also linear (Fig. 2).

The enzyme is not inactivated during the measurements and substrate saturation subsists, i.e. since we are dealing here with a heterogeneous system, excess substrate surface is accessible during the measurement of activity (Figs. 3 and 4).

By means of the reciprocal plotting of reaction rate and substrate concentration according to Lineweaver and Burk the maximum reaction rate and the Michaelis constant of the enzyme were determined (Fig. 5).

The lipase preparation of NBC was used as a model enzyme for the kinetic tests.

The method is suitable for measuring the enzyme concentration of various lipase preparations, that is for the comparison of such preparations, provided enzyme concentration and reaction time are chosen in such a way that the experimental results are within the linear part of the curve of hydrolysis.

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## APPLICATION OF FILMING INHIBITORS TO THE PREVENTION OF CORROSION OF STEAM- HEATED FOOD EQUIPMENT CONTAINING ALSO LARGE AMOUNTS OF AIR

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The main cause of corrosion in (water + steam + condensate) systems is their oxygen and carbon dioxide content. One way of preventing corrosion would be the removal or binding of oxygen and carbon dioxide. In the case studied steam mixed with air is an important medium of heat transfer in the equipment to be protected, thus only those methods of corrosion prevention can be applied in which the corrosive agent is not removed, but its contact with the metal surface is prevented in some other way. Filming amines seem to be suitable for the insulation of the metal surface.

Corrosion degradation of carbon steel specimens, under conditions simulating those prevailing in the industrial equipment to be protected, was investigated in a specially constructed experimental apparatus under laboratory conditions. Next any reduction in corrosion due to the application of filming inhibitors was investigated. It appeared that, with an appropriately chosen dosage, about 70% protection can be achieved within a relatively short period of time. Starting from this experience, the inhibitor was subjected to plant trials.

In the course of these plant experiments it was observed that in the *Hunister* hydrostatic sterilizer the complicated water-steam-air system causes very serious corrosion. For some weeks after its application the filming inhibitor displayed no, or hardly any protective action, but once the inhibitor layer was formed on the surfaces to be protected in the equipment, that is the surfaces became saturated, a marked drop in corrosion took place. The decrease in wall thickness of the specimens, calculated from their weight loss, was at the end of the experiment 0.08 to 0.3 mm per year. At the end of an appr. eight months long plant trial a dark grey, water repellent layer was found on the internal surfaces of the equipment; the amount of rust was negligible.

These plant experiments proved that with the application of a filming inhibitor very substantial protection against corrosion can be achieved under conditions and with dosages differing from those described in the available literature.

Corrosion of systems containing water, steam and condensate is a problem which affects all branches of the food industry, since most food plants have their own steam generating plant and only in exceptional cases do they receive steam through pipe-lines from another plant. Boiler plant equipment is designed to have a long useful life and is a metal construction of high value which in case of corrosion damage may cause the breakdown of the entire plant and even explosion. Hence, appropriate corrosion protection of such equipment is an extremely important problem.

Investigating the processes taking place in boilers WICKERT and PILZ (1950) found that only at temperatures around and above 500 °C will a chemical reaction occur between the metal and steam, thus this cannot be the



fundamental process responsible for corrosion. WILDE (1968) claims that in hot water the corrosion rate of steel is reduced by the presence of ammonia, increased by the presence of oxygen up to a certain concentration limit, while hydrogen has no effect. It follows unequivocally from practical observations and from the reports of a number of authors (MAGUIRE, 1954; KAHLER & BROWN, 1954; DALBKE, 1956; BASS & SINDERY, 1957; BRINDISI, 1959) that carbon dioxide is one of the main causes, and oxygen only a secondary cause of corrosion. Carbon dioxide causes general thinning of the walls, filiform corrosion and cavitations and, in addition, acidification of the boiler feed water. Oxygen leads mainly to pitting.

Consequently, to prevent the corrosion of steam and condensate systems, steam has to be freed of its carbon dioxide and oxygen content and even of other non-condensing gases, since these have a highly detrimental effect on the heat transfer factor.

The first step of degassing can be performed by the known thermal procedure and the small quantities of residual oxygen and carbon dioxide can then be removed, preferably by means of some chemical reaction. One of the most widely applied methods for the removal of oxygen is treatment with hydrazine where water and nitrogen are formed according to the following equation:



Another frequently used method consists in binding the residual oxygen of soft and thermally de-aerated boiler feed water by leading it through a bed of iron shavings.

POURBAIX's electrochemical experiments have furnished evidence on the passivity of iron in the pH range between 8.5 and 10 (BÁCSKAI & HORVÁTH, 1964). Since by raising the pH it is possible to passivate the metal of the boiler and of its fittings and, in addition, carbon dioxide can also be bound in this way, by an appropriate rise of the pH of boiler feed waters it is possible to suppress corrosion substantially.

It is obvious that only those methods of alkalification will lead to the desired result which ensure the same favourable alkaline pH in the steam space of the boiler as in the feed water. The application of the simplest volatile base, i.e. of ammonia seemed to be an evident solution, and was in fact a good one, since ammonia causes no caustic embrittlement, the necessary quantity changes only with the carbon dioxide content of the boiler feed water and it is highly stable at usual boiler temperatures (ANDRES, 1954; HÖMG, 1956).

Beside these favourable features the application of ammonia has the drawback of strongly attacking copper or copper alloy occasionally incorporated in certain parts of the system to be protected.



If instead of ammonia volatile amines are used, all the advantages of corrosion protection by means of ammonia can be achieved without the accompanying drawbacks (KOVÁCS, 1960; TUCK & OSBORN, 1960; HARRISON, 1962; TINLEY, 1964).

The amines most widely used for the neutralization of boiler feed waters are cyclohexylamine and morpholine. These amines are volatile with steam and react with the carbon dioxide to form compounds.

Thus corrosion can be greatly suppressed by the chemical binding of oxygen and carbon dioxide, as well as by the adjustment of pH to a favourable value. These protective measures are, however, lengthy and cumbersome.

The search for a simpler method of protection which can be applied more easily led finally to KAHLER's patent (1949) which relates to the preparation of a corrosion protective substance and to a protective procedure which is a simplification of the above protective measures and achieves with a single substance the same result for which several substances and operations had been needed earlier. His method involves the application of filming amines by means of which corrosion can be reduced to a minimum.

Filming amines are saturated aliphatic amines with long straight carbon chains and contain in the case of primary amines at least 12, but preferably 18 carbon atoms. In secondary and tertiary amines each of the two or three carbon chains contains 12 or more carbon atoms (BÁCSKAI, 1969).

Octadecylamine or stearylamine ( $C_{18}H_{37}NH_2$ ) is the most frequently used and best known filming amine. This, like the other filming amines, is a water insoluble wax-like substance. Its addition to the steam system is rather cumbersome, thus, recently, dispersions with favourable properties, such as suitability for dilution with water, were developed.

Investigation of the protective mechanism of filming amines led to the finding that this mechanism is entirely different from that found with the neutralizing amines discussed above. Long carbon chain, polar amines settle on the surface of the metal and the free electron pair of their nitrogen atom forms a bond with the metal so that the carbon chain will be perpendicular to the metal surface (KAJANNE, 1957). This monomolecular layer forms a water repellent film which acts as an efficient barrier against the aggressive medium, thereby preventing corrosion (TINLEY, 1964). In contrast with neutralizing amines, filming amines do not react with carbon dioxide.

In order to achieve the desired protection by the application of filming amines, KAHLER and BROWN (1954) claim that 15 to 30 ppm of commercial grade octadecylamine, dispersed in the steam produced, is needed. KAJANNE (1957) used in his experiments 2 g of pure octadecylamine for 1 metric ton of steam and achieved 97% protection. Today the manufacturers of filming amine inhibitors specify the quantities to be added.



Filming amines have a property which will cause the regeneration of used and corroded surfaces. Since the free electron pair of the nitrogen of the amine is capable of forming a bond with the metal, by penetrating through the pores of the corrosion product to the surface after a certain time it will, so to speak, wrench off these products. If the filming amine is added in adequate quantities, after the removal of the corrosion products corrosion will be quite slight.

Several investigators agree that the corrosion preventing action of filming amines is excellent even under plant conditions (KAHLER & BROWN, 1954; KAJANNE, 1957). The degree of protection is independent of the quantity of oxygen and carbon dioxide present in the steam (HARRISON, 1962; ANON, 1965).

None of the authors mentions any disadvantageous properties of filming amines.

From the aspect of applicability in the food industry, in addition to the above described properties, the toxicity of the substance is also of paramount importance. KAHLER and BROWN (1954) found that, in animal experiments, doses hundred times higher than detected in steam are still not toxic and do not attack the skin. Thus in countries in which octadecylamine is extensively used, its application in the food industry, except the dairy and meat industries, is also permitted. In the dairy and meat industries the steam may come into direct contact with the food to be consumed and animal fats are inclined to accumulate octadecylamine.

Because of their favourable properties, we have chosen one of the filming amines when the necessity arose to protect the *Hunister* against corrosion, a complicated problem which could not be solved by any of the other known methods. The results of the related experiments can, of course, be utilized in the corrosion protection of other systems, e.g. of commonly used steam and condensate systems.

The *Hunister* is a dual-system, divided hydrostatic sterilizer used in the canning industry. It is based on a Hungarian Patent and ensures sterilization conditions quite different from those usual in earlier sterilizers also operating by the hydrostatic principle (OTT, 1971). To reach the temperature needed for sterilization of canned goods the *Hunister* has a very complicated water-steam-air system available. The apparatus consists of six water columns connected in series and six air, or steam-air mixture columns, resp., keeping hydrostatic balance with the water columns and serving to heat the cans to be sterilized. This is followed by the sterilizing space in which the temperature must not exceed 135 °C and the pressure 2.4 atmospheres, and then by another six water and steam-air mixture columns which are the mirror image of the first six and serve to cool the heat-treated cans. At the temperature of sterilization this complicated water-steam-air system is highly

corrosive. In solving the corrosion protection of the fairly large apparatus made of carbon steel the following conditions had to be borne in mind:

a) considering the size of the apparatus and the quantity of steel needed for its construction, the use of corrosion resistant structural material is not feasible, due to economic reasons;

b) application of a protective coating cannot be realized for constructional, cost and efficiency reasons;

c) chemical de-aerating agents, generally used in boiler plants, e.g. hydrazine or neutralizing amines, cannot be applied, since air is indispensable in the apparatus, so that the binding or neutralization of its components cannot be attempted;

d) under these conditions the application of filming amines seemed the only feasible solution, thus these formed the subject of our experiments.

## 1. Materials and methods

### 1.1. Materials

1.1.1. *Structural material.* In the experiments 50·100·1 mm specimens made of carbon steel sheets quality A III 23 (Hungarian Standard MSz 23) were used. They were polished and degreased with trichloroethylene. In the laboratory experiments the specimens were suspended on glass hooks. In the plant experiments 30·50·4 mm specimens, made of boilerplate KI 2 (Hungarian Standard MSz 1741) were used. The specimens were hung on a special hard-wood rod and placed at various points in the apparatus.

1.1.2. *Inhibitor.* Octadecylamine marketed under the trade name "Armofilm" by Armour Hess Chemicals, Ltd., Leeds, was used as filming inhibitor in the experiments. The most important properties of this inhibitor are, according to the Information Sheet of the manufacturer, as follows:

Appearance: white, pearly, mobile dispersion.

Specific gravity at 20 °C: 0.92.

Drop point: 0 °C.

Viscosity at 22 °C: 900 cp.

1.1.3. *Reagent for the qualitative detection of the inhibitor.* For the qualitative detection of octadecylamine a citrate buffer, pH 3.5 and eosin indicator were used.

The eosin indicator was prepared by adding 10 ml of a 100 mg per litre eosin solution to 90 ml of 1,1,2,2-tetrachloroethane, the developed colour was eliminated by the addition of 0.5 g of citric acid and the solution filtered through filter paper (COATES, 1960).



*1.1.4. Reagents for the determination of iron.* Iron content of the condensate of the experimental equipment was determined by means of the method of SPANYÁR and KEVEI (1961) using the following reagents:

Concentrated hydrochloric acid to effect the dissolution of colloidal iron; Ascorbic acid, analytical grade; 2% solution of  $\alpha,\alpha'$ -dipyridyl; 20% solution of ammonium acetate.

## *1.2. Methods*

*1.2.1. Laboratory corrosion test equipment.* Figure 1 shows the diagram of the apparatus used for laboratory tests.

The apparatus consists of a pressure-tight, electrically heated vessel which is connected to a coil pipe cooled on the outside with water. This cooler ensures a forced circulation of the system and leads the condensed water back to the bottom of the pressure-tight vessel. The temperature of the latter is automatically controlled with the help of a contact thermometer and fitted with a pressure gauge and a safety valve. The lid of the apparatus is fixed by means of bolts which can be released and inside the apparatus a support shelf with glass hooks in an appropriate position serves to hold the samples.

*1.2.2. Corrosion tests.* The specimens pretreated in accordance with para. 1.1.1 were weighed on the analytical balance up to the fourth decimal, the pressure vessel was filled with 5 litre of tap water, 10 specimens were half immersed in water and other 10 placed in the vapour space. The lid was then fixed, the vessel heated, with the valve open, to 100 °C, the valve closed and the temperature raised to 140 °C.

Thorough de-aeration was not among the objectives of the experiment, since in the hydrostatic sterilizer, too, the corrosive action of air has to be reckoned with.

The duration of the experiment was calculated from the moment when operation temperature had been reached.

*Experiments without inhibitor:* In order to establish the changes in steel caused by water and steam of some given temperature under the experimental conditions, the specimens were exposed to corrosive attacks of various durations. At the end of the experiment the corrosion products were mechanically removed from the surface of the specimens and after visual inspection the weight losses were determined.

*Experiments in the presence of inhibitor:* In this case an inhibitor solution was added to the feed water of the pressure vessel. Corrosion tests were later modified by adding the inhibitor after de-aeration by means of a pump. In one series of experiments the inhibitor was added daily, in another at hourly intervals. Each dose provided a concentration of 10 mg per litre of initial feed water.

The protective action of the inhibitor was calculated by the following formula:

$$IP \% = \frac{W_0 - W}{W_0} \cdot 100$$

where

$IP\%$  = percentage protective action of inhibitor

$W_0$  = weight loss of specimens without inhibitor

$W$  = weight loss of specimens in the presence of inhibitor.

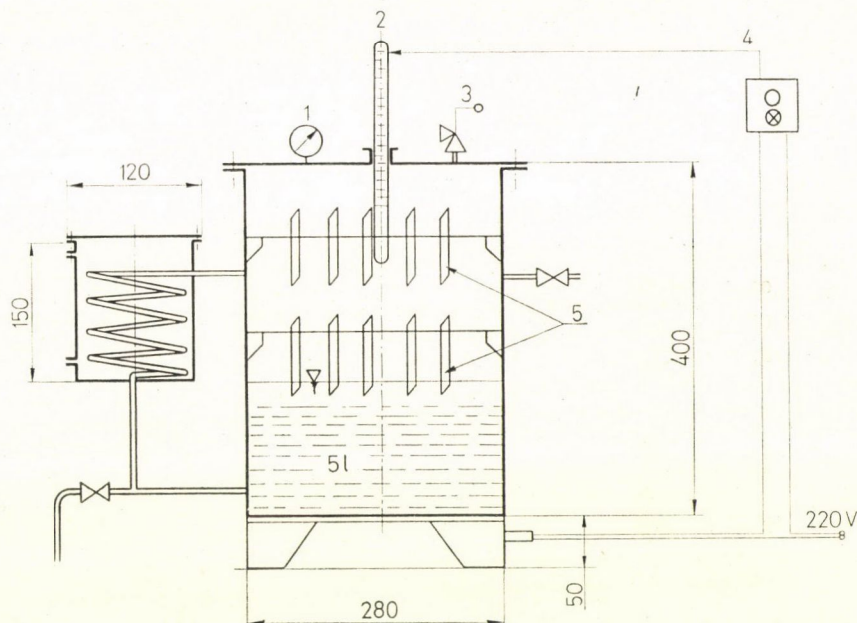


Fig. 1. Diagram of the laboratory test apparatus. 1. Pressure gauge. 2. Contact thermometer. 3. Safety valve. 4. Relay. 5. Specimens. Dimensions are expressed in mm.

*1.2.3. Detection of the presence of inhibitor.* The inhibitor was detected in the condensate formed in the apparatus, i.e. in the circulating cooling water. Five millilitres of condensate, 2 ml of the eosin indicator and 0.2 ml of the pH 3.5 citrate buffer were measured into a measuring cylinder provided with a ground glass stopper and the mixture was thoroughly shaken. After separation of the aqueous from the solvent phase, the pink tinge of the solvent phase indicated the presence of octadecylamine.

*1.2.4. Determination of iron dissolved in the condensate.* The condensate sample was thoroughly shaken to ensure as much as possible an even distribution of colloidal iron and an adequate quantity (in case of visibly high iron content 2 to 5 ml, in case of lower iron contents 10, 20 or 30 ml) was pipetted



into a 100 ml volumetric flask and the colloiddally dissolved iron hydroxide brought into solution by the addition of a few drops of concentrated hydrochloric acid. The flask was then filled up to the mark, an aliquot part transferred to a 50 ml volumetric flask and the ferric ions reduced to ferrous ions by means of ascorbic acid. 2 ml of the  $\alpha, \alpha'$ -dipyridyl solution and 10 ml of the 20% ammonium acetate solution were then added to the contents of the flask, if necessary, the pH adjusted to 4 to 5 with concentrated ammonia and the flask filled up to the mark. After the development of colour, which took roughly one hour, the extinction of the samples was measured at 496 nm with the spectrophotometer "Spektromom 360."

Iron content was calculated as follows:

$$\text{iron content mg/l} = \frac{285.7 \cdot E \cdot V}{d \cdot S \cdot v}$$

where

$E$  = extinction value

$V$  = total volume of stock solution

$d$  = thickness of the cell

$S$  = quantity of condensate added to the stock solution

$v$  = quantity of stock solution used in the colorimetric reaction.

*1.2.5. Mathematical statistical evaluation of the experimental results.* The numerical data of the measurements were subjected to statistical analysis. The mean values, standard deviation and coefficient of variation were calculated.

The progress of the corrosion process in time was investigated by means of Student's  $t$  test, after having ascertained with the help of the  $F$  test in each case that there is no significant difference in deviations and only if this condition was fulfilled was the  $t$  test applied.

## 2. Results

### 2.1. Results of laboratory tests

*2.1.1. Corrosion of carbon steel specimens without inhibitor.* The specimens described in para. 1.1.1 were exposed in the corrosion test apparatus of para. 1.2.1 to corrosive attacks lasting for different lengths of time.

The changes observed after exposure can be summarized as follows.

In case of specimens half-immersed in water the part under water suffered less damage with only few visible spots and most part of the surface intact. The corrosion of the part protruding from the water was similar to that observed on specimens in the vapour phase. A boundary line appeared at water level, but the changes at this boundary line were apparently not more marked than the changes on parts of the specimen which extruded into the vapour phase.

Almost the entire surface of the specimens exposed to the vapour space was covered by a black corrosion product which became thicker with time. This corrosion product could be easily removed by abrasion, leaving on the metal surface point-like and circular spots. Weight loss of the specimens was determined after the removal of the corrosion product. Average weight loss of the specimens, standard deviation and coefficient of variation after various periods of exposure are given in Table 1 on the basis of 10 parallel measurements.

Table 1

*Weight loss of specimens, half-immersed in the feed water and placed in the steam phase of the experimental equipment, as a function of time*

Averages of 10 parallel measurements

Duration of test, days	Weight loss of half-immersed specimens			Weight loss of specimens in the steam phase		
	Average g/m <sup>2</sup>	Standard deviation g/m <sup>2</sup>	Coefficient of variation %	Average g/m <sup>2</sup>	Standard deviation g/m <sup>2</sup>	Coefficient of variation %
2	0.90	0.16	18.27	1.34	0.43	31.65
3	2.30	0.34	14.76	2.33	1.08	46.25
4	1.91	0.23	12.18	5.44	1.33	24.59
8	1.32	0.15	11.76	3.76	1.00	26.54
12	0.83	0.20	24.41	5.54	1.04	18.73

### 2.1.2. Corrosion of carbon steel specimens in the presence of the inhibitor.

Experiments similar to those described in para. 2.1.1 were performed with the modification that, to reduce corrosion, the inhibitor Armofilm was introduced into the system.

In the first experiment the inhibitor was added daily in a quantity of 10 mg per 1 litre of feed water. It was found after five days, in the course of which no steam was removed, that the protective layer produced by the inhibitor developed only in spots on the surface, so that protection was incomplete, as confirmed by the values of weight losses.

In the second experiment the period of the test was increased to 12 days with all other conditions unchanged. It seemed that during this time the entire surface of the specimen became covered with the protective layer; weight loss was nevertheless fairly high.

In the third experiment 10 mg of inhibitor per litre of feed water were added hourly into the apparatus. The presence of filming amine was detected in the steam sample already on the third day of the experiment, indicating a saturation of the system with the inhibitor. From then on the inhibitor was added every hour for eight hours per day and no inhibitor was added for the next 16 hours. This procedure was repeated each day.



After 12 days the specimens half-immersed in water were practically metal-clean, with not more than traces of corrosion visible on their surface.

From the surface of specimens exposed to corrosion in the steam phase the water-repellent layer of the inhibitor was difficult to remove, the specimens had to be washed with solvent. On the surface of the cleaned specimens, mainly near the area of the hooks, a bluish-grey oxide layer was formed and in the pitches of the surface some traces of corrosion were detected, but a considerable part of the surface was intact.

Average weight loss, standard deviation and coefficient of variation are given for various application methods of the inhibitor and for various periods of attack in Table 2 based on 10 parallel measurements.

Table 2

*Effect of the method of inhibitor addition on the weight loss of specimens*  
Averages of 10 parallel measurements

Addition		Weight loss of specimens					
Method	Duration, days	half-immersed in water			in steam		
		Average g/m <sup>2</sup>	Standard deviation g/m <sup>2</sup>	Coefficient of variation %	Average g/m <sup>2</sup>	Standard deviation g/m <sup>2</sup>	Coefficient of variation %
Repeated daily	5	0.85	0.087	10.23	2.34	0.55	23.55
Repeated daily	12	4.29	0.83	19.23	7.64	1.94	25.41
Repeated hourly	12	0.36	0.13	26.42	1.60	0.41	39.28

The protective action of the inhibitor in the steam space can be calculated from the results of laboratory experiments using the data in the last lines of Tables 1 and 2.

$$IP \% = \frac{5.54 - 1.60}{5.54} \cdot 100 = 71.11 \sim 71 \%$$

## 2.2. Plant experiments

*2.2.1. Condition of the equipment prior to the experiment.* After a few days of trial operation considerable corrosion appeared on the internal surface of the sterilizing equipment. A pale, loose, spongy rust was formed on the internal surface of the walls, a considerable part of this rust came off and accumulated on the bottom of the equipment. It is quite probable that part of the rust found on the internal surface of the equipment prior to the experiment had been formed before trial operations were started, most likely during the construction of the equipment. During trial operations the iron content of the condensate and cooling water was rather high, 207.1 mg/litre, not counting the rust which had settled on the bottom.

*2.2.2. Dosage of the inhibitor.* An inhibitor feed tank was mounted on the equipment and dosage performed manually. In contrast with the method described in the literature, the quantity of added inhibitor was not proportional to the produced (fed) quantity of steam, since in the case of the *Hunister* this value varies, but was proportional to time, that is a given quantity of inhibitor was added into the hottest space of the equipment hourly.

It was observed that as the inhibitor was added the rust gradually disappeared from the surface and after about six weeks the iron content of the condensate and cooling water was not more than 8.5 mg per litre, and after two months only 1.23 mg/litre.

*2.2.3. Checking of corrosion rate by means of specimens.* Corrosion due to corrosive attack in the equipment was further followed by measuring the weight loss of specimens exposed in five different places of the equipment to corrosive attack. Series of five times 20 specimens of carbon steel plate quality A III. 23 (Hungarian Standard MSz 23) were placed into the equipment before the addition of the inhibitor had begun. It was expected that changes in corrosion with time could be traced by inspecting five specimens each after 6, 12, 18 and 24 weeks.

It was found after the first six weeks, when the first 5 specimens were inspected, that they were damaged to a degree which necessitated the reducing of the full exposure period to 12 weeks. After 12 weeks all the remaining specimens were inspected and after removal of the corrosion product their weight loss was determined.

The data are summed up in Table 3, including the reduction in wall thickness in mm per year, calculated from the rate of weight losses.

Table 3

*Average weight loss of carbon steel specimens placed at points of different temperatures in the sterilizing equipment, standard deviation and coefficient of variation of the results of 5 and 15 parallel measurements, resp., and calculated decrease in wall thickness (mm/year) immediately after starting inhibitor treatment*

Range of exposure temperature °C	After six weeks exposure				After twelve weeks exposure			
	Weight loss			DWTH <sup>3</sup> mm/year	Weight loss			DWTH <sup>3</sup> mm/year
	Average g/m <sup>2</sup>	S <sup>1</sup> g/m <sup>2</sup>	CV <sup>2</sup> %		Average g/m <sup>2</sup>	S <sup>1</sup> g/m <sup>2</sup>	CV <sup>2</sup> %	
128—130	435	73	16.85	0.48	502.9	56.9	11.31	0.28
106—110	1883	335	17.79	2.09	** —	—	—	—
86—90	2317	261	11.26	2.58	2223.8	393.8	18.74	1.22
66—70	816	295	36.50	0.90	1207.3	390.3	34.21	0.66
56—60	1396	169	12.11	1.55	1374.9	190.3	18.64	0.75

<sup>1</sup> Standard deviation

<sup>2</sup> Coefficient of variation

<sup>3</sup> Decrease of wall thickness

\*\* The specimens could no longer be evaluated



The data obtained at two different times were subjected to statistical analysis. There was no significant difference in the weight loss values when the specimens were exposed to steam spaces of 56° to 60 °C and 86° to 90 °C for six and twelve weeks, resp., but significant difference at the 99.9% probability level was found between the specimens exposed for six and twelve weeks, resp., to steam spaces of 66° to 70 °C and of 128° to 130 °C (Specimens exposed for twelve weeks to steam of 106° to 110 °C could no longer be evaluated). The results of the calculations are shown in Table 4.

Table 4

*Analysis of variance of the data obtained at two different times (Table 3) by means of the  $F$  and  $t$  tests*

Range of exposure temperature °C	$S_1^2$	$S_2^2$	$F_{\text{calculated}}$	$F_{95\%}$	DF	
					$V_1$	$V_2$
128–130	0.0484	0.0292	1.66	3.11	14	4
86–90	0.6155	1.3958	2.27	5.84	4	14
66–70	0.7852	1.3714	1.75	5.84	4	14
56–60	0.2583	0.3262	1.26	5.84	4	14

$v_1$  = numerator,  $v_2$  = denominator

Since the  $F$  test shows no significant difference the deviations originate from the same population, the  $t$  test can be performed.

Range of exposure temperature °C	$n_1$	$n_2$	$S_d$	$t_{\text{calculated}}$
128–130	5	15	0.0944	4.155
86–90	5	15	0.5709	0.948
66–70	5	15	0.3309	6.846
56–60	5	15	0.2880	0.043

$t_{95\%} = 2.101$ ;  $t_{99\%} = 2.878$ ;  $t_{99.9\%} = 3.922$ ;  $DF = 18$

$n_1$  = number of parallels

$n_2$  = number of parallels

$S_1$  = standard deviation of the first statistical population

$S_2$  = standard deviation of the second statistical population

$S_d$  = common standard deviation

The experiments were repeated with new specimens (K1 2, Hungarian Standard 1741). It was expected that the comparison of weight losses of specimens exposed for two different periods of time to corrosive attack would provide information on the progress of the corrosion process. Because of plant

conditions, the specimens were kept for 5 and 8 weeks, resp., at various points of the equipment. A slight incrustation was observed on the specimens after both five and eight weeks of exposure (natural, non-softened water was used in the equipment for cooling), but corrosive changes were slight. Weight losses obtained after the removal of the corrosion products and calculated for unit surface area are given in Table 5.

Table 5

*Average weight loss of boiler steel plate specimens placed at points of different temperatures in the sterilizing equipment, standard deviation and coefficient of variation of the results of ten parallel measurements as well as the calculated wall thickness decrease in mm/year. Experiment started four months after the addition of the inhibitor*

Range of exposure temperature °C	After five weeks exposure				After twelve weeks exposure			
	Weight loss			DWTH <sup>3</sup> mm/year	Weight loss			DWTH <sup>3</sup> mm/year
	Average g/m <sup>2</sup>	S <sup>1</sup> g/m <sup>2</sup>	CV <sup>2</sup> %		Average g/m <sup>2</sup>	S <sup>1</sup> g/m <sup>2</sup>	CV <sup>2</sup> %	
128—130	259.01	55.74	21.52	0.34	360.60	70.19	19.46	0.30
86—90	140.57	23.98	17.05	0.19	179.67	30.60	17.03	0.15
66—70	166.84	30.58	18.32	0.22	82.25	29.59	35.97	0.07
56—60	105.44	29.04	27.54	0.14	179.67	35.79	19.92	0.15
26—30	132.17	22.00	16.65	0.18	99.72	13.21	13.25	0.08

<sup>1</sup> Standard deviation

<sup>2</sup> Coefficient of variation

<sup>3</sup> Decrease of wall thickness

Statistical analysis indicates a highly significant difference between the specimens exposed for two different periods of time, thus corrosion has progressed at a certain rate (Table 6).

It appears from the reduction of wall thickness that corrosion rate is comparatively low, since by this time the equipment was saturated with the inhibitor and this latter was in fact effective.

*2.2.4. Appearance of the internal surface of the equipment at the end of the experiment.* At the end of the approximately eight months long period of the experiment it was found that from that part of the internal surface of the equipment which was in contact with steam, rust was removed and a dark grey, almost black deposit similar to an oxide layer was formed. This layer was greasy to the touch and water repellent. Some small rust spots were found on the internal wall exposed to steam at temperatures of 128° to 130 °C.



Table 6

*Analysis of variance of the data obtained at two different times (Table 5) by means of the  $F$  and  $t$  tests*

Range of exposure temperature °C	$S_1^2$	$S_2^2$	$F_{\text{calculated}}$	$F_{95\%}$	DF	
					$V_1$	$V_2$
128-130	0.0412	0.0653	1.58	3.8	9	9
86-90	0.0076	0.0124	1.63	3.8	9	9
66-70	0.0124	0.0116	1.06	3.29	7	9
56-60	0.0112	0.0170	1.51	3.8	9	9
26-30	0.0064	0.0023	2.78	3.18	9	9

$v_1$  = numerator,  $v_2$  = denominator

Since the  $F$  test led to no significant difference the deviations originate from the same population, the  $t$  test can be performed.

Range of exposure temperature °C	$n_1$	$n_2$	$S_d$	$t_{\text{calculated}}$
128-130	10	10	0.103	8.00
86-90	10	10	0.047	6.73
66-70	10	8	0.049	13.02*
56-60	10	10	0.055	10.90
26-30	10	10	0.031	8.47

$t_{95\%} = 2.101$ ;  $t_{99\%} = 2.878$ ;  $t_{99.9\%} = 3.922$ ;  $DF = 18$ ; \* =  $DF = 16$

$n_1$  = number of parallels

$n_2$  = number of parallels

$S_1$  = standard deviation of the first statistical population

$S_2$  = standard deviation of the second statistical population

$S_d$  = common standard deviation

### 3. Conclusions

#### 3.1. Conclusions of the laboratory experiments

Corrosion of carbon steel proceeds at different rates in hot water and in steam. After 48 hours only faint spots appeared on the under-water parts of specimens half immersed in water, the major part of the surface was bright. This is the natural consequence of the escape of corrosive gases from water as temperature rises.

In these experiments, mainly the phenomena occurring at the boundary of the two phases were investigated. It was found that during the period

of the experiment the damage under water and on the interface was not as serious as in the steam phase.

The corrosive action of steam results in the formation of black corrosion products of loose structure on the surface of the specimens. Thin layers of this product were velvety, thicker layers had a spongy structure. After removal of the corrosion product circular and point like cavities were visible on the surface of the metal. After a longer period of exposure these cavities tended to cover the entire surface of the specimen. The initial, rather high corrosion rate decreased with the progress of time and, though due to the porous structure of the corrosion product the process continued, its rate was considerably lowered.

The observations during the addition of the filming inhibitor have led to the following conclusions:

When the inhibitor was added once daily, the protective coating on the surface of the specimens developed only in spots during a five-day period, indicating that under the given experimental conditions this amount of inhibitor was not sufficient to saturate the surfaces. For this purpose more inhibitor or a longer period of dosage was required. On the other hand it might be assumed that an inhibitor added once a day may cause the formation of a protective layer which, however, is liable to damage within a relatively short time, and has no chance of regeneration in the 24-hour interval in which no inhibitor is added. The first supposition seems the more probable one and the results of 12 day experiments appear to confirm it.

The results obtained with adding the inhibitor once a day for 12 days showed that 12 days are sufficient for the formation of the inhibitor layer, since after this period a water repellent layer covering the entire metal surface was found, but weight loss data seem to point to the formation of a coherent inhibitor layer only immediately before the end of the experiment, as shown by the greater damage on specimens in earlier periods of the trial.

In the experiment in which the addition of the inhibitor was repeated hourly, good results were obtained, proving that continuous replacement of the inhibitor is essential from the point of view of efficient protection. The 71% protection which was achieved in this way can be considered satisfactory and quite possibly protection efficiency can be further improved by a more prolonged application of the inhibitor.

The inhibitor film caused the surface of specimens to turn dark grey and water repellent.

After the removal of the inhibitor film the metal surface had an even dark grey colour, was bright, free from cavities, thus the inhibitor in fact afforded good protection to the specimens.

On the basis of these laboratory findings the inhibitor was judged suitable for plant trials which were subsequently performed.



### 3.2. Conclusions of the plant trials

On the corrosion of the hydrostatic sterilizer *Hunister* in the absence of the inhibitor only observations and no numerical data are available.

Prior to the beginning of the dosage of the filming inhibitor traces of serious corrosion had been detected on the walls of the equipment and considerable rust accumulation at its bottom. This rust was probably formed partly during the construction and partly during the trial operations of the equipment.

Soon after the beginning of inhibitor addition the iron content of the condensate and cooling water commenced to drop gradually and finally reached a relatively low value. The rust removed from the surface by the first doses of inhibitor were cleaned from the bottom of the equipment, after which no further rust accumulated there during the whole period of the trial.

Weight loss of the specimens led to the conclusion that immediately after the beginning of inhibitor application corrosion rate was still very high, but as the surface became gradually saturated with the inhibitor, this rate dropped and when a second series of specimens were exposed, their decrease in wall thickness was considerably lower than that of the members of the first series.

Despite the comparatively short period of the experiment, the data of the plant trial indicate that the particular filming inhibitor, Armofilm, provided good corrosion protection to the hydrostatic sterilizer *Hunister* in which steam with high air content and untreated cooling water were used, when the inhibitor was added proportionally to time, thus under conditions quite different from those described in the literature.

The economic advantage of this method of corrosion inhibition is supported by the results of preliminary calculations, according to which the total cost of this type of corrosion protection of the *Hunister* sterilizer over 10 years is not more than 1.5% of its investment cost.

Application of the filming inhibitor may lead to greatly reduced corrosion and thus to considerable savings in other branches of the food industry, too.

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## INVESTIGATIONS INTO THE POSSIBILITIES OF ENZYMATIC HYDROLYSIS OF CELLULOSE CONTAINING WASTES

### PART II — CHEMICAL PRETREATMENT OF POPLAR SAWDUST AND OF CORN COB TO IMPROVE THE EFFICIENCY OF ENZYMATIC HYDROLYSIS

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In order to improve the possibilities of the enzymatic hydrolysis of cellulose containing wastes several methods of chemical pretreatment were tried, including digestion with dilute sulphuric acid and various procedures of delignification (treatment with hydrochloric acid — dioxan — water mixtures, or with sodium hydroxide solutions, etc.). Sulphuric acid digestion was applied to poplar sawdust and corn cob, delignification to sawdust raw materials. The efficiency of digestion with sulphuric acid was determined by measuring the quantity of dissolved reducing substance, while the degree of delignification was determined from the weight loss of sawdust and qualitatively detected by means of hydrochloric phloroglucinol.

Our main findings were as follows:

1. If a suspension containing 5 g of sawdust in 100 ml of 1 to 5% w/v sulphuric acid is kept at 100°, 120° and 133 °C, resp., for 2 hours, 4.5 to 10.5% of the solids content of sawdust will be converted into reducing substances. From the aspect of the convenience of operations and on the basis of the results described in the first part of our series of communications, we found that treatment at 100 °C with 2% w/v sulphuric acid is the most advantageous, so that subsequent experiments were performed under these conditions, when 5.5% of the sawdust was converted into reducing sugar at a sawdust: sulphuric acid ratio 1 : 0.4.

2. The quantity of dissolved reducing substance increases with increasing concentration of the sawdust suspension. In case of a sawdust concentration of 10% w/v 6.8% of the dry matter content of sawdust is converted into reducing sugar. The sugar content of the hydrolysis liquor is 0.63% w/v which is about 2.5 times higher than the one formed in a sawdust suspension of 5% w/v concentration while specific sulphuric acid utilization is reduced to about half (1 : 0.2).

3. Pretreatment of the 5 and 10% suspensions of corn cob with 2% sulphuric acid at 100 °C for 2 hours, caused 29–30% of the solids content to go into solution.

4. Delignification with hydrochloric acid — dioxan — water mixtures was most efficient on adding 5 N HCl and dioxan (4 : 6 and 1 : 9, resp.) in a ratio of 100 : 5, to the sawdust suspension. 46–47% of the sawdust could be dissolved by a 3-hour treatment at 92 °C and the qualitatively detectable lignin content dropped to a minimum value. The weight loss is about twice the lignin content of poplar sawdust, indicating that in the course of this treatment considerable quantities of other substances had also gone into solution.

5. The quantity of sawdust dissolved by NaOH increases with the concentration of the alkali up to a 15% NaOH concentration. The degree of delignification is lower than in the case of hydrochloric acid — dioxan mixtures.

6. In the range below 1 mm the grain size of sawdust has but a slight influence on the efficiency of delignification.

The results of the enzymatic hydrolysis of chemically pretreated sawdust and corn cob will be reported in a forthcoming paper.

In our previous communication (VÁMOS-VIGYÁZÓ *et al.*, 1972) we have described the results of preliminary experiments in which poplar sawdust was subjected to hydrolysis by sulphuric acid and cellulase enzyme. By



treating a neutralized, 5% w/v suspension of sulphuric acid prehydrolyzed sawdust with cellulase for 12 hours, a liquor containing 1.63% w/v of reducing substance was obtained, thus 26.5% of the sawdust was converted into reducing substance.

The total cellulose and hemicellulose content of poplar wood is about 77% (NIKITIN, 1955), thus it was supposed that the efficiency of the method could be improved by an appropriate modification of the hydrolysis process. Consequently, we wanted to subject the effect of both chemical and enzymatic treatment on the hydrolyzability of sawdust to a closer scrutiny. In the present paper we shall discuss the results obtained by chemical pretreatment.

The objects of the experiments were:

- to reduce the quantity of sulphuric acid used in preliminary hydrolysis and to simplify the operation of pretreatment;
- to raise the concentration of sawdust suspensions;
- to make the cellulose and hemicellulose content of sawdust more readily accessible to the enzymes by means of the reduction of the lignin content.

We have extended our investigations beside those on sawdust to corn cob which is more liable to hydrolysis.

## 1. Materials and methods

### 1.1. Materials

*1.1.1. Poplar sawdust.* The fraction between 0.315 and 1.00 mm of sawdust fractionated by means of a set of vibrating screens was used for pretreatment with sulphuric acid. For pretreatments of other types (alkaline, with dioxan, etc.) smaller fractions, namely those between 0.125 and 0.315 mm, and those smaller than 0.125 mm were also included. The solids content of sawdust was determined prior to each series of experiments by drying to constant weight at 105 °C.

*1.1.2. Corn cob.* The grain sizes of the available ground corn cob were determined by sieve analysis. The distribution according to grain size was:

smaller than 0.200 mm	9.4%
0.200 to 0.400 mm	11.6%
0.400 to 0.630 mm	13.0%
0.630 to 0.800 mm	11.4%
0.800 to 1.000 mm	16.4%
1.000 to 1.250 mm	10.6%
1.250 to 1.600 mm	20.4%
greater than 1.600 mm	6.8%

In the experiments the entire air-dry milled product was used whose moisture content was determined by drying to constant weight in the drying cabinet. Moisture content was 6.8%.

*1.1.3. Materials of pretreatment.* For the pretreatment of sawdust sulphuric acid solutions of 1 to 5% w/v concentrations, mixtures of various proportions of hydrochloric acid, dioxan and water, sodium hydroxide, sodium salicylate and sodium benzoate solutions of various concentrations were used.

## *1.2. Methods*

### *1.2.1. Pretreatment with sulphuric acid*

*1.2.1.1. Pretreatment of sawdust with sulphuric acid.* — Five g of air-dry sawdust was suspended in 100 ml of sulphuric acid of 1, 2, 3, 4 and 5% w/v, concentrations, respectively, and the suspension was kept at 100, 120 and 133°C in the autoclave for 2 hours. Suspensions containing 7.5 and 10 g of sawdust in 100 ml were also pretreated with 2% sulphuric acid at 100 °C. More concentrated suspensions could not be prepared as, because of the extreme swelling of sawdust it was impossible to ensure homogeneous wetting and mixing.

*1.2.1.2. Pretreatment of corn cob with sulphuric acid.* — Corn cob suspensions of 5 g per 100 ml and of 10 g per 100 ml were treated at 100°C with 2% (w/v) sulphuric acid for 2 hours.

### *1.2.2. Delignification*

*1.2.2.1. Pretreatment of sawdust with a mixture of hydrochloric acid, dioxan and water.* — Experiments were carried out with mixtures of hydrochloric acid, dioxan and water, too, in order to remove the lignin content of sawdust (LENGYEL & MORVAY, 1965).

The proportions of analytical grade 5 *N* HCl, dioxan and water in the mixtures are shown in Table 1.

Mixtures Nos 3, 5 and 7 had a hydrochloric acid concentration of 2 *N*, those of Nos 2, 4 and 6 a HCl concentration of 0.5 *N*. When calculating the weight per cent of the components their specific gravities were taken as 1 ( $D_4^{20}$  of 36% HCl = 1.180, of dioxan = 1.035) (NÉMETH, 1943; RAUEN, 1964).

100 ml of these solutions each were added to 5 g samples of sawdust, the mixtures carefully sealed and kept at 92 °C under reflux for 3 hours. After cooling each mixture was washed first with 2500 ml of hot and then with 2500 ml of cold distilled water into a dried and weighed sintered glass crucible G3 and the crucible with the material dried at 105 °C to constant weight. From the weight differences the quantity of material dissolved by treatment was calculated.



Table 1

*Composition of mixtures containing 5 N HCl, dioxan and distilled water*

No.	5 N HCl	Dioxan	Distilled water	Weight per cent of hydrochloric acid, dioxan and distilled water in the mixture
	part(s) by weight in 10 parts by weight of the mixture			
1	—	10	—	0 : 100 : 0
2	1	9	—	1.8 : 90 : 8.2
3	4	6	—	7.2 : 60 : 32.8
4	1	6	3	1.8 : 60 : 38.2
5	4	3	3	7.2 : 30 : 62.8
6	1	3	6	1.8 : 30 : 68.2
7	4	0	6	7.2 : 0 : 92.8

*1.2.2.2. Alkaline pretreatment of sawdust.* — To each 5-g sample of sawdust 100 ml of a NaOH solution of 1, 3, 5, 15 and 45% concentration, resp., was added. Treatment was carried out at 100 °C, for 3 hours. Determination of the quantity of sawdust dissolved by this procedure was the same as that described in para. 1.2.2.1.

*1.2.2.3. Pretreatment of sawdust by other methods.* — It was tried to delignify sawdust by treatment with sodium salicylate and sodium benzoate, resp. (LENGYEL & MORVAY, 1965). To 5 g samples of dry sawdust 1.0, 3.0 and 10.0 g, resp., of the above substances were added and the mixtures suspended in 100 ml of distilled water. The suspension was allowed to stand at 100 °C for 3 hours.

*1.2.2.4. The effect of the grain size of sawdust on pretreatment.* — According to relevant literature (PEW & WEYNA, 1962) from the aspect of efficiency of alkaline treatment the grain size of sawdust plays an important role.

By the application of three sawdust fractions we extended this test to other methods of pretreatment. The grain size of the

fine fraction (F) was	<0.125 mm,
of the medium fraction (M)	0.125 to 0.315 mm,
of the coarse fraction (C)	0.315 to 1.00 mm.

The three fractions were treated with 1 : 9 and 4 : 6 mixtures of 5 N HCl: dioxan (see para. 1.2.2.1) and also with 1 and 15% NaOH (see para. 1.2.2.2).

*1.2.2.5. Detection of lignin content.* — The lignin content of sawdust was detected with a 1 : 1 mixture of 2% alcoholic phloroglucinol and concentrated HCl (SÁRKÁNY & SZALAY, 1957). Sawdust moistened with hydrochloric acid-alcohol was used as control and change in colour was recorded in comparison with the control. The degree of colouring was marked with crosses: one cross

represents a colour slightly deviating from that of the control, two crosses faint appearance of a red tinge, three crosses vivid red colour.

*1.2.2.6. Determination of reducing substance content.* — The efficiency of sulphuric acid pretreatment was measured by the quantity of reducing substance expressed in glucose as recommended by SOMOGYI (1952). Prior to the determination of the reducing substance content the filtered liquors were freed of proteins (BALDWIN *et al.*, 1953). In some cases beside the reducing substance content the conversion value is also shown. This latter gives the percentage solids content in sawdust which has been converted into reducing substance.

### *1.2.3. Mathematical statistical evaluation of the results*

The results were statistically evaluated by means of Student's *t* test. The Figures include the significance levels of the deviations between the compared values (KÖRMENDY, 1964). Significant differences at the 95, 99 and 99.9% probability levels were marked with 1, 2 and 3 x-signs, respectively.

## **2. Results**

### *2.1. Pretreatment with dilute sulphuric acid*

*2.1.1. Pretreatment of sawdust with dilute sulphuric acid.* The effect of the concentration of sulphuric acid and of the temperature on the quantity of reducing substance obtained from a 5% w/v sawdust suspension is shown in Fig. 1. The Figure includes the conversion values.

At 100° and 120 °C the reducing substance content rises significantly with increasing sulphuric acid concentration up to 2%, but a further rise in concentration causes no significant change. At 133 °C in the concentration range between 1 and 3% of sulphuric acid practically identical quantities of reducing matter are formed, but these quantities are significantly lower when concentration is raised to 4 and 5%.

In the sulphuric acid concentration range between 1 and 3% the reducing substance content rises significantly with increasing temperature from 100 °C up to 133 °C, but when the concentration is 4 and 5%, this rise is manifest only up to 120 °C.

Depending upon the concentration of sulphuric acid in the sawdust suspensions, 0.21 to 0.33 g of reducing substance per 100 ml of suspension was formed at 100 °C, 0.54 to 0.82 g at 120 °C and 0.76 to 0.92 g at 133 °C, corresponding to 4–7%, 11–18% and 16–20% conversion of the solids content of sawdust into reducing sugar.

The relationship between the concentration of the sawdust suspension and the quantity of reducing substance formed in the course of pretreatment with 2% sulphuric acid at 100 °C for 2 hours is illustrated in Fig. 2.



Reducing substance content rises very highly significantly with increasing sawdust concentration, but the value of conversion (since for the different sawdust concentrations the reference value is also different) differs only in case of the suspension of the highest concentration (10% w/v) from the other two values.

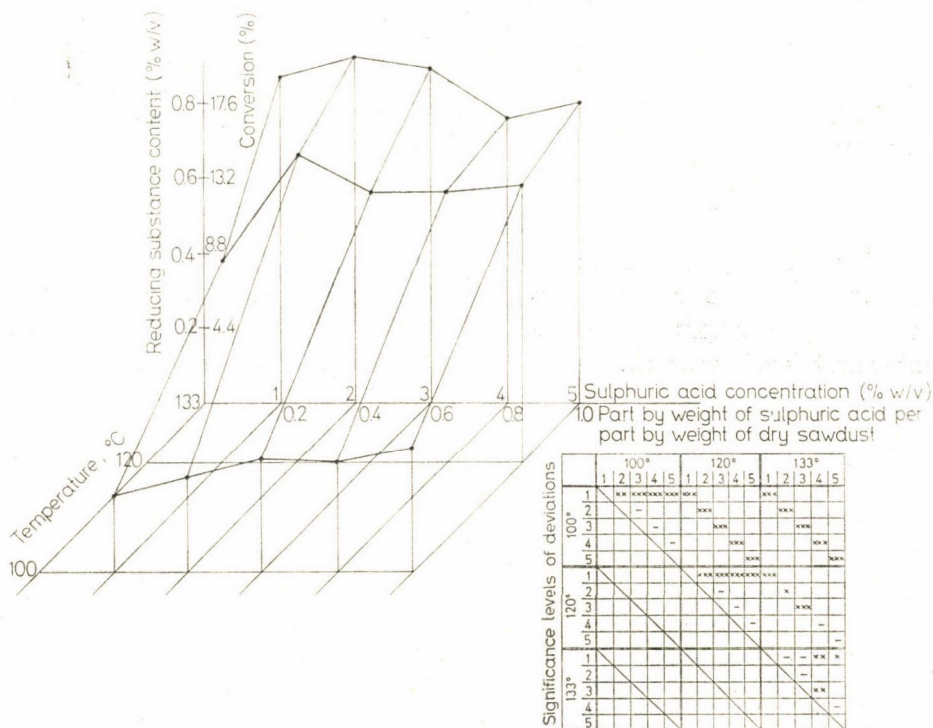


Fig. 1. Effect of temperature and of sulphuric acid concentration on the hydrolysis of poplar sawdust. Concentration of sawdust suspension: 5% w/v. Duration of treatment: 2 hours

**2.1.2. Hydrolysis of corn cob with dilute sulphuric acid.** When suspensions of 5 and 10 g of air-dry corn cob in 100 ml are hydrolyzed with 2% sulphuric acid at 100 °C for 2 hours 1.4 and 2.76 g, respectively, of reducing substance per 100 ml is formed. These values correspond to 30.5 and 29.8% conversion, respectively.

Thus corn cob is considerably more liable to hydrolysis with dilute sulphuric acid than poplar sawdust.

## 2.2. Delignification

**2.2.1. Pretreatment of sawdust with mixtures of hydrochloric acid, dioxan and water.** Fig. 3 shows the effect of various ternary mixtures and also of 2 N

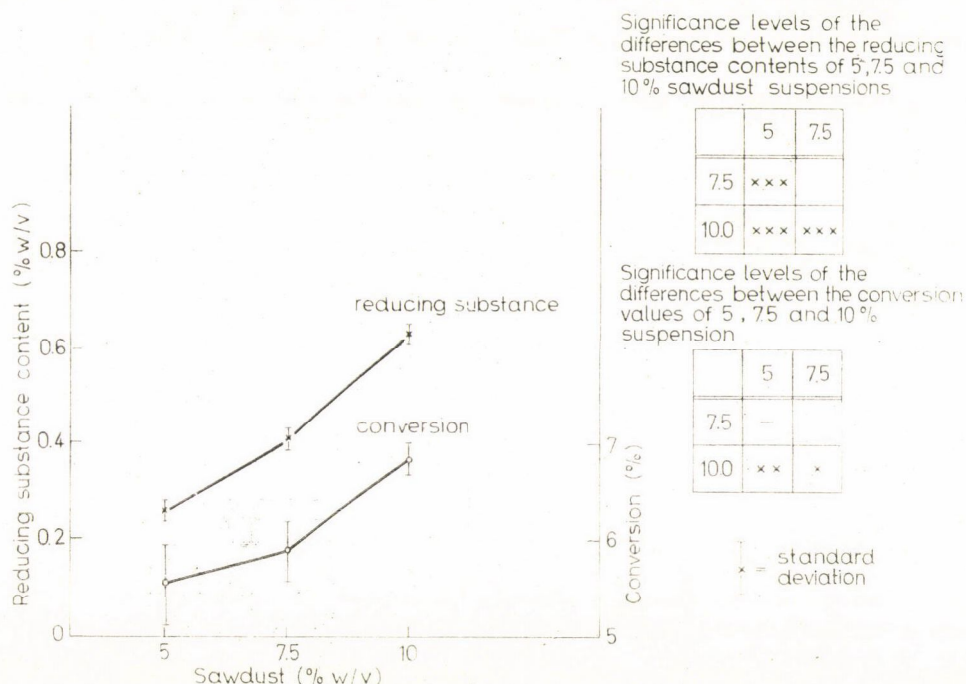


Fig. 2. Effect of the concentration of sawdust suspension on the quantity of dissolved reducing substance. Sulphuric acid concentration: 2% w/v. Temperature: 100°C. Duration of treatment: 2 hours

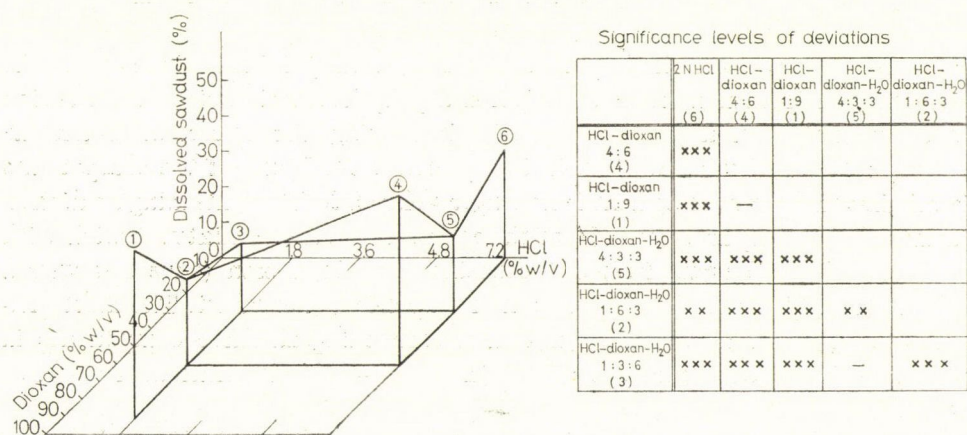


Fig. 3. Effect of hydrochloric acid + dioxan mixtures of various ratios on the quantity of dissolved sawdust. Concentration of sawdust suspension: 5% w/v. Temperature: 92°C. Duration of treatment: 3 hours



HCl (without dioxan) on sawdust, that is the percentage of dissolved sawdust vs. the dioxan and hydrochloric acid content of the mixtures. The Figure does not include the quantity of sawdust dissolved by dioxan alone, since this is negligible.

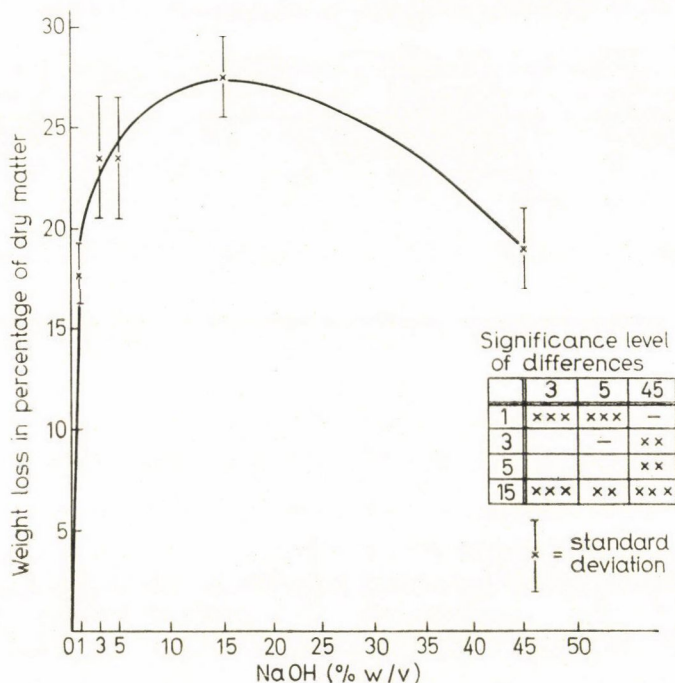


Fig. 4. Effect of the concentration of the NaOH solution on the quantity of dissolved sawdust. Concentration of sawdust suspension: 5% w/v. Temperature: 100 °C. Duration of treatment: 3 hours. Grain size of sawdust: 0.125 to 0.315 mm

It appears from the Figure that when ternary mixtures are used, the efficiency of treatment rises with increasing hydrochloric acid concentration at a constant dioxan content, and with increasing dioxan content when the concentration of hydrochloric acid is kept constant. If one of the components is missing, the above statement is no longer valid: 2 *N* HCl containing no dioxan (mixture No. 7) hydrolyses 30% of the sawdust, while the mixture which with respect to HCl is again 2 *N*, but contains 30% of dioxan (thus less water, mixture No. 5) causes the dissolution of only 24% of sawdust. The most efficient of all solutions were Nos 2 and 3, containing all three components in rather different concentrations; with them 46–47% dissolution of sawdust was achieved.

**2.2.2. Alkaline pretreatment of sawdust.** The quantities of sawdust dissolved by NaOH solutions of various concentrations are shown in Fig. 4 in percentages of the initial quantity of sawdust.

It appears from the Figure that with NaOH concentrations increasing up to 15% the quantity of dissolved sawdust increases, but with 45% NaOH practically the same quantity of sawdust can be dissolved as with 1%.

Significance level of differences

		HCl-dioxan						NaOH					
		2:3			1:9			1%			15%		
		F	M	C	F	M	C	F	M	C	F	M	C
HCl-dioxan	2:3	—	xx	—	—	—	—	—	—	—	—	—	—
	1:9	—	—	—	xx	xx	—	—	—	—	—	—	—
	NaOH	—	—	—	—	—	—	—	—	—	xxx	xxx	xxx
NaOH	1%	—	—	—	—	—	—	—	—	—	xxx	xxx	xxx
	15%	—	—	—	—	—	—	—	—	—	xxx	xxx	xxx
		—	—	—	—	—	—	—	—	—	—	—	—

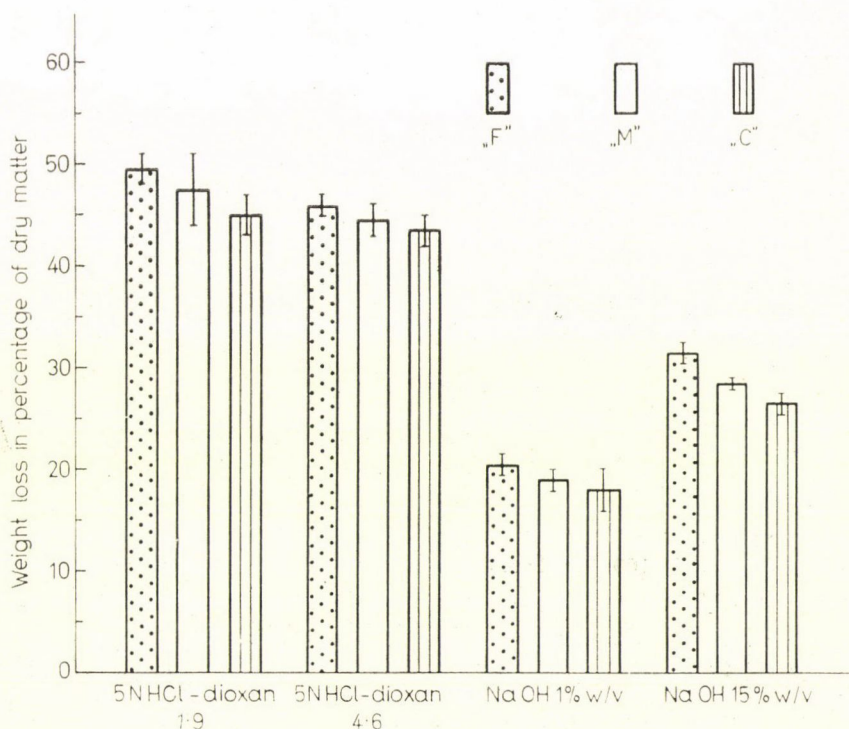


Fig. 5. Effect of the grain size of sawdust on the quantity of sawdust dissolved by hydrochloric acid — dioxan and NaOH treatment, resp. Granulometry: Fine fraction (F) < 0.125 mm. Medium fraction (M) 0.125 to 0.315 mm. Coarse fraction (C) 0.315 to 1.00 mm

1 % NaOH solution caused the dissolution of 18% of the sawdust, 15% NaOH solution that of 27.5% of sawdust. In the first case the quantity of sawdust dissolved by 1 g of NaOH is 0.9, in the second case 0.09 g, thus the specific efficiency of the dilute NaOH solution is ten times higher.



When prior to treatment with 15% NaOH 2% sulphuric acid is applied at 100 °C for 2 hours, 33% of the sawdust can be brought into solution, which is a significantly higher quantity than that achieved by 15% NaOH alone.

*2.2.3. Other methods for the pretreatment of sawdust.* When with reference to sawdust 20 to 200% of sodium salicylate or 60 and 200% of sodium benzoate was applied, not more than 1 to 5% of the sawdust went into solution, while 20% of sodium benzoate caused the dissolution of about 10% of sawdust. Change of pH from 7.5 to 3.0 resulted in yields which were not significantly different.

*2.2.4. Effect of grain size on the quantity of sawdust dissolved by pretreatment.* According to the results illustrated in Fig. 5 only more drastic treatments will lead to significant differences in the quantity of dissolved sawdust in case of the three investigated grain sizes, but this difference is not great. For instance, 15% NaOH causes the dissolution of 27% of the "coarse" fraction and of 31.5% of the "fine" fraction, while with hydrochloric acid-dioxan-water mixtures the corresponding values are 45–49.5% and 43.5–46.0%, resp.

*2.2.5. Changes in lignin content due to treatment.* All investigated sawdust samples show a certain colour change compared to the colour of the moistened control, when tested with phloroglucinol dissolved in hydrochloric acid. As shown in Table 2 untreated sawdust gives a bright red colour (3 crosses)

Table 2

*Detection of lignin in sawdusts by means of phloroglucinol dissolved in hydrochloric acid after various pretreatments*

Method of pretreatment	Weight loss in % of dry matter	Colour reaction
Untreated	0.51	+++
2% H <sub>2</sub> SO <sub>4</sub> , 100 °C, 2 hours	6.01	+++
2% H <sub>2</sub> SO <sub>4</sub> + 15% NaOH	33.11	++
1% NaOH	19.33	++
3% NaOH	23.49	++
5% NaOH	23.53	++
15% NaOH	27.53	++
45% NaOH	18.95	++
5 N HCl + dioxan (1 : 9)	47.66	+
5 N HCl + dioxan (4 : 6)	47.44	+

+++ bright red

++ pale red

+ slight discolouration

Reagent: 2% phloroglucinol in alcohol + concentrated HCl (1 : 1). Colour was compared to a blank of sawdust moistened with alcohol + conc. HCl (1 : 1)

and the colour reaction of the sample treated with 2% sulphuric acid is quite similar.

Weight losses of samples pretreated with NaOH vary between 20 and 30%, their reaction is quite faint. They are marked with 2 crosses.

In cases when pretreatment caused a 50% drop in the weight of sawdust, colouring was hardly noticeable and these samples were marked with 1 cross.

### 3. Conclusions

Examination of the results of the different chemical pretreatments from the aspect of the objectives put forward in the Introduction has led to the following conclusions:

#### 3.1. Pretreatment with dilute sulphuric acid

The 5 to 8% conversion produced by sulphuric acid and found advantageous according to preliminary experiments from the aspect of the efficiency of enzymatic treatment (VAMOS *et al.*, 1970) could be realized by keeping 5% w/v sawdust suspensions with 2% sulphuric acid at 100 °C for 2 hours (Fig. 1). In this way the two-step hydrolysis used in the preliminary experiments (ANON, 1970) was replaced by a one-step procedure, having the same time requirement, with a simultaneous decrease in sulphuric acid requirement to one quarter of the original.

With increasing concentration of the sawdust suspension a significant rise in the reducing substance content of the liquor was achieved: in case of a 10% w/v sawdust suspension the value of conversion was between 6 and 7%. This value is still in the range in which the subsequent enzymatic hydrolysis was found to be efficient. Higher sawdust concentration has a twofold advantage: on the one hand, it facilitates a further reduction in specific sulphuric acid consumption (in case of a 10% w/v suspension by 50%), and on the other hand, pretreatment leads to a liquor of higher reducing substance content. Doubling of the sawdust concentration may lead to a 150% increase in the reducing substance content of the liquor (Fig. 2).

Corn cob was found to be more liable to hydrolysis with dilute sulphuric acid. At 100 °C and with 2% sulphuric acid 29 to 30% conversion both of the 5 and 10% w/v suspensions were attained in 2 hours. Probably in case of this raw material, too, a substantially lower conversion value is sufficient to ensure the efficiency of enzymatic treatment which perhaps can be realized with still less sulphuric acid or in a shorter period of time.



### 3.2. Reduction of lignin content

Dilute sulphuric acid treatment had practically no effect on the lignin content of sawdust (Table 2).

Of the other treatments carried out with the definite aim of delignification treatment with sodium hydroxide or with sulphuric acid — sodium hydroxide reduced comparatively slightly the intensity of the lignin reaction. Concentration of the alkali had no effect on the intensity of the lignin reaction. Consequently of the alkaline treatments that with more dilute (1%) NaOH appears to be more favourable, not only because of its lower alkali requirement, but also because solutions of higher concentration cause a 25 to 27% dissolution of the sawdust. The lignin content of poplar is not more than about 22% (NIKITIN, 1955), thus alkaline treatment results in a dissolution of other substances, too (presumably first of all of hemicelluloses) which are then lost from the aspect of further processing. Also in the literature we find reports on the application of fairly dilute, 0.5, 1 and maximum 8%, alkali solutions (PEW & WEYNA, 1962).

From the point of view of delignification the mixtures 5 N HCl — dioxan are the most efficient. These caused, however, a dissolution of almost the half of the solids content of sawdust, roughly twice the quantity corresponding to the total lignin content. It seems probable that in the sawdust treated in this manner the polymers less accessible to hydrolysis had been left behind, so that the enzymatic hydrolysis of the delignified residue is not very promising.

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## BEHAVIOUR OF POLYPHOSPHATES DURING THE STORAGE OF MEAT PRODUCTS

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(Received March 24, 1972)

Hydrolysis of diphosphate (pyrophosphate) and triphosphate (tripolyphosphate) was followed during the storage of comminuted or cured, non-comminuted post rigor semimembranosus pork muscle at 4 °C. A considerable hydrolysis of both polyphosphates took place under these conditions. Toluene, which was added to avoid bacterial growth, had an accelerating effect on hydrolysis of polyphosphates due presumably to plasmolysis. NaCl seemed to activate pyrophosphatase and inhibit tripolyphosphatase. The rate of hydrolysis of both polyphosphates was lower in cured, non-comminuted meat.

Polyphosphates are widely used in the meat industry of several countries. As generally known, they improve the water binding capacity of meat products. According to FUKAZAWA *et al.* (1961) diphosphate (pyrophosphate) causes the dissociation of actomyosin to actin and myosin. YASUI *et al.* (1964) established that only diphosphate molecules were effective on actomyosin. Triphosphate (tripolyphosphate) had an effect only after its hydrolysis to diphosphate.

There are very few data on the behaviour of polyphosphates during the ripening of meat emulsions and cured meat. Recently NAKAMURA *et al.* (1969) found two pyrophosphatases in rabbit skeletal muscle. Acid pyrophosphatase was found to be associated with muscle particulate components, a neutral one was present in the soluble fraction.

### 1. Materials and methods

#### 1.1. Test material and its preparation

Semimembranosus muscle was obtained from post rigor pork carcasses. The separable fat and connective tissue were removed and the muscle was ground in a meat grinder. 2.0% (w/w) NaCl, and 0.5% (w/w) polyphosphate ( $\text{Na}_4\text{P}_2\text{O}_7$  or  $\text{Na}_5\text{P}_3\text{O}_{10}$ ) were added to the ground tissue and thoroughly mixed. The samples were kept in a refrigerator at 4 °C.

To investigate the behaviour of polyphosphates in cured meat, the semimembranosus muscles were pumped to 110% weight with curing brine containing 20% (w/w) NaCl, 0.1% (w/w)  $\text{NaNO}_2$  and 5% (w/w) polyphosphate ( $\text{Na}_4\text{P}_2\text{O}_7$  or  $\text{Na}_5\text{P}_3\text{O}_{10}$ ) and stored in refrigerator at 4 °C.



### 1.2. Extraction of the phosphates

Extraction of samples was carried out with cold trichloroacetic acid at 4 °C according to the method of POHJA *et al.* (1957).

### 1.3. Separation and determination of phosphates

Separation of phosphates was made by thin-layer chromatography on carboxymethyl cellulose MN 300 with the solvent isopropanol: 20% (w/w) trichloroacetic acid: water: concentrated ammonia (70 : 20 : 10 : 0.3) at 10 °C. The phosphate content of the eluted spots was measured according to the method of KARL-KROUPA (1956).

To avoid bacterial growth generally toluene was added to the meat samples.

## 2. Results and conclusions

### 2.1. Effect of storage and toluene on the hydrolysis of phosphates

Table 1 shows the breakdown of diphosphate and triphosphate as a function of the storage time at 4 °C. As shown total hydrolysis of diphosphate occurred in 3 days. The hydrolysis of triphosphate is a consecutive reaction. It is interesting to note, that a considerable amount of triphosphate (40 – 50%) hydrolyses immediately after addition to the minced meat. It was

Table 1

*Breakdown of diphosphate and triphosphate in minced post rigor semimembranosus pork muscle at 4 °C (pH = 5.8–6.2)*

Storage time (days)	2% NaCl and 0.5% $\text{Na}_4\text{P}_2\text{O}_7$ added		2% NaCl and 0.5% $\text{Na}_5\text{P}_3\text{O}_{10}$ added			
	Without toluene	With toluene	Without toluene		With toluene	
	PP%	PP%	TP%	PP%	TP%	PP%
0	100.0 ± 0.8	100.0 ± 1.1	40.6 ± 2.4	39.6 ± 2.3	31.0 ± 2.4	46.0 ± 3.2
1	70.0 ± 3.6	50.5 ± 5.0	12.7 ± 3.0	28.7 ± 2.4	5.8 ± 2.4	42.2 ± 2.2
2	3.0 ± 1.0	6.0 ± 1.2	4.9 ± 2.0	23.3 ± 3.0	4.2 ± 1.0	31.0 ± 2.4
3	0	0	4.2 ± 2.1	12.4 ± 2.0	3.0 ± 1.2	15.7 ± 2.2
4			0	0	0	0

TP = triphosphate

PP = diphosphate

Average of 4 determinations

established that this phenomenon is not due to the hydrolysing effect of trichloroacetic acid during extraction of the sample. Trichloroacetic acid hydrolyses only about 10% of the total amount which was added to the meat. The rapid hydrolysis of TP is in some special cases somewhat similar to the ATP breakdown in muscular tissue. It is also interesting to note that toluene, which reduced total bacterial count by about two logarithmic units, seems to have a significant accelerating effect on the hydrolysis of diphosphate. The phenomenon may be explained by plasmolysis i.e. by releasing pyrophosphatase isozyme which was associated with particulate components of the tissue.

### 2.2. Effect of NaCl on the hydrolysis of phosphates

Table 2 shows the effect of NaCl (2% w/w) on the rate of hydrolysis of the two polyphosphates. As shown NaCl had a considerable inhibiting effect on the rate of hydrolysis of diphosphate. On the contrary, NaCl seemed to have an activating effect on triphosphatase.

Table 2

*Effect of 2% (w/w) NaCl on the rate of hydrolysis of polyphosphates in minced post rigor semimembranosus pork muscle at 4°C (pH = 5.9)*

Storage time (days)	0.5% $\text{Na}_4\text{P}_2\text{O}_7$ added		0.5% $\text{Na}_5\text{P}_3\text{O}_{10}$ added			
	Without NaCl	With NaCl	Without NaCl		With NaCl	
	PP%	PP%	TP%	PP%	TP%	PP%
0	100.0±0.45	100.0±0.57	56.2±1.6	29.2±1.7	39.0±0.9	40.3±1.5
1	0	44.0±2.0	4.7±1.7	11.8±1.5	9.2±2.1	9.6±1.6
2		0	0	0	0	0

TP = triphosphate

PP = diphosphate

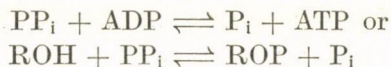
Average of 4 determinations

### 2.3. Hydrolysis of phosphates in comminuted and non-comminuted meat

Table 3 shows the results obtained with cured semimembranosus muscle. It is obvious that the rate of hydrolysis of polyphosphates is lower in non-comminuted meat.

Experiments on the subcellular distribution of di- and triphosphatase are not yet finished. It has been proved that sarcoplasmic tri- and diphosphatase isozymes do exist in pork meat. YASUI *et al.* (1964) found only myosin B triphosphatase in muscular tissue. It should be noted that the breakdown of pyrophosphate, apart from hydrolysis, could follow other reaction patterns, too:





(Where ROH = hydroxyl group of a monosaccharide,  $\text{PP}_i$  = pyrophosphate,  $\text{P}_i$  = orthophosphate). This makes experimentation on reaction kinetics more difficult.

Table 3

*Rate of hydrolysis of  $\text{Na}_4\text{P}_2\text{O}_7$  and  $\text{Na}_5\text{P}_3\text{O}_{10}$  in cured, non-comminuted post rigor semimembraneus muscle ( $2H = 6.1$ )*

Storage time (days)	Meat pumped with $\text{NaCl}$ , $\text{NaNO}_2$ and $\text{Na}_4\text{P}_2\text{O}_7$	Meat pumped with $\text{NaCl}$ , $\text{NaNO}_2$ and $\text{Na}_5\text{P}_3\text{O}_{10}$	
	PP%	TP%	PP%
0	$100.0 \pm 0.3$	$21.2 \pm 1.5$	$52.5 \pm 1.5$
1	$69.6 \pm 2.1$	$9.4 \pm 1.9$	$41.0 \pm 1.8$
2	$50.4 \pm 2.5$	$8.0 \pm 2.5$	$35.5 \pm 2.0$
3	$24.0 \pm 3.0$	$7.3 \pm 1.8$	$13.1 \pm 1.8$
4	0	0	0

TP = triphosphate

PP = diphosphate

Average of 2 determinations

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## KINETIC ANALYSIS OF DIAUXIC GROWTH OF ASPERGILLUS AWAMORI

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(Received April 20, 1972)

The growth of an *Aspergillus awamori* strain proved to be diauxic when cultivated in media containing corn-steep-liquor and pectin or sucrose as the carbon sources. In media containing pectin as the carbon source without corn-steep-liquor diauxic has not occurred, while in media containing no corn-steep-liquor but sucrose as the carbon source diauxic growth was also found.

The growth of the above cultures was kinetically analysed by the method of KONO and ASAI. The specific growth rate ( $k$ ) of the cultures grown in pectin-containing media with and without corn-steep-liquor were 0.192, 0.104 and 0.134, resp. while in sucrose-containing media the specific growth rate of the cultures with and without corn-steep-liquor were 0.126, 0.034 and 0.117, 0.054, resp.

The reason for the occurrence of diauxie in corn-steep-liquor-containing media appears to be the assimilation of amino acids of corn-steep-liquor origin in the first part of the diauxic cycle. In the second part the assimilation of galacturonic acid and reducing sugars, decomposed by the enzymes of the culture from pectin and sucrose resp., took place.

In media containing sucrose and no corn-steep-liquor the reason for diauxie is supposed to be the difference in the rate of glucose and fructose assimilation.

Industrial cultivation of microorganisms, and the increased application of continuous processes made it necessary to study the growth kinetics of microorganisms (MONOD, 1949; GADEN, 1955; HUMPHREY *et al.*, 1966; AIBA *et al.*, 1967). Diauxic growth of microorganisms was studied on the basis of chemical reaction kinetics described by KONO and ASAI (1971).

Diauxic growth was also observed during the submerged cultivation of several *Aspergilli*. The reasons for the occurrence of diauxic growth of *Aspergillus awamori* under different cultivation conditions are analysed in the present paper.

### 1. Materials and methods

An *Aspergillus awamori* strain was cultivated in 10-litre glass fermenters, with an aeration rate of 1 vol/vol·min and a speed of agitation of 460 rpm. Oxygen solution rate was 51 mmol O<sub>2</sub>/l·h. Temperature of incubation was 28 °C.

*Composition of the various media used was as follows:*

*Medium No. 1.* Pectin 30 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 7 g, KH<sub>2</sub>PO<sub>4</sub> 2 g, corn-steep-liquor 10 g/1000 ml, pH: 4.5;



*Medium No. 2.* Identical with Medium No. 1 but not containing corn-steep-liquor;

*Medium No. 3.* Sucrose 70 g,  $(\text{NH}_4)_2\text{SO}_4$  20 g,  $\text{KH}_2\text{PO}_4$  2 g, corn-steep-liquor 10 g/1000 ml, pH: 3.5;

*Medium No. 4.* Identical with the previous medium but without corn-steep-liquor.

*Inoculum:* 600 ml of a 24-hour culture was added in each case to 6 litre medium.

*Mycelial weight* of the culture was determined after drying to constant weight in an oven at 105 °C.

*Calculations* of the kinetic analysis were made with an electronic digital computer (ODRA-1204).

As a locating reagent p-amino-phenol was used. The solvent system for the detection of galacturonic acid oligomers was as follows: butanol-acetic acid-water (5 : 2 : 3) while 0.5% bromophenol-blue was used as a locating reagent. Samples were applied to the sheets in volumes of 1 and 2  $\mu\text{l}$  for sugars and organic acids, resp.

## 2. Results

Growth, and growth rate of *Aspergillus awamori* culture in media containing pectin as the carbon source and corn-steep-liquor are given in Fig. 1.

As can be seen in the figure, the growth of the above microorganism has shown typical diauxie in both cases when the mycelial weight was plotted against time of cultivation, as well as when the rate of growth was plotted against mycelial concentration.

The kinetic constants of the above culture are given in Table 1.

Data of growth calculated with the values of the kinetic constants (determined graphically from Fig. 1) are marked on the growth curve by crosses.

Growth and rate of growth of *Aspergillus awamori* in media containing pectin as carbon source without corn-steep-liquor are given in Fig. 2.

Figure 2 clearly shows that diauxic growth was not found in *Aspergillus awamori* cultures when media not containing corn-steep-liquor were used. The growth curve of the microorganism shows the usual form and is composed of four stages.

The kinetic constants of the culture are given in Table 2.

Culture growth calculated by the use of kinetic constants, marked on the growth curve with crosses, is shown in Fig. 2.

Growth of the *Aspergillus awamori* culture was also diauxic when grown in corn-steep-liquor-containing media with sucrose as the carbon source (Fig. 3).

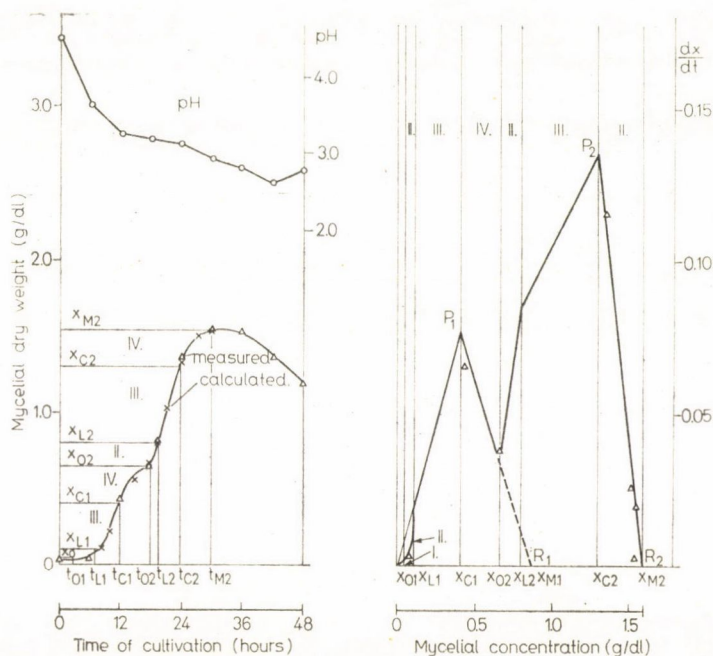


Fig. 1. The growth of *Aspergillus awamori* in medium No. 1 containing corn-steep-liquor and pectin as the carbon source. Relationship between cell concentration and growth rate. The slope of line  $\overline{OP}$  represents the growth rate ( $k$ ) of the exponential phase

Table 1

Kinetic constants of *Aspergillus awamori* grown in media containing corn-steep-liquor and pectin as carbon sources  
(Fig. 1)

Part of diauxie	$t_0$	$t_L$	$t_C$	$t_M$	$x_0$	$x_L$	$x_C$	$x_M$	$k$
1.	3	7.5	12	20	0.03	0.10	0.40	0.88	0.192
2.	18	20.5	23	36.5	0.66	0.80	1.30	1.60	0.104

#### Nomenclature

$k$  = growth rate constant ( $\text{hr}^{-1}$ )

$t$  = time (h)

$x$  = cell concentration (g/dl)

#### Subscripts

- 0 refers to the boundary of an induction phase and a transient phase
- L refers to the boundary of a transient phase and an exponential growth phase
- C refers to the critical point, that is the boundary of an exponential growth phase and a declining growth phase
- M refers to theoretical maximum value of the cell concentration



Table 2

Kinetic constants of *Aspergillus awamori* grown in media containing pectin as carbon source and no corn-steep-liquor  
(Fig. 2)

$t_0$	$t_L$	$t_C$	$t_M$	$x_0$	$x_L$	$x_C$	$x_M$	$k$
3	4.5	16.5	36	0.03	0.10	0.70	1.0	0.134

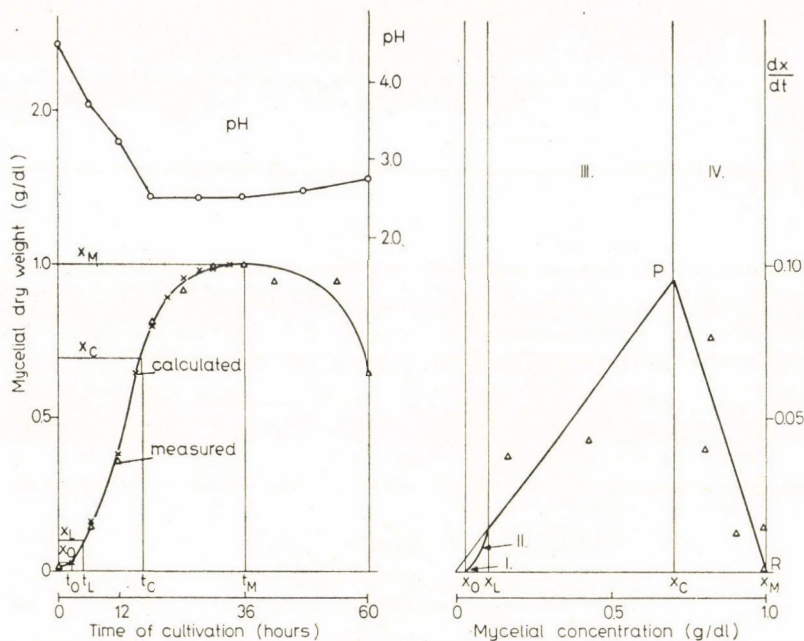


Fig. 2. The growth of *Aspergillus awamori* in medium No. 2 containing pectin as the carbon source and no corn-steep-liquor. Relationship between cell concentration and growth rate

Table 3

Kinetic constants of *Aspergillus awamori* grown in media containing corn-steep-liquor and sucrose as carbon sources  
(Fig. 3)

Part of diauxie	$t_0$	$t_L$	$t_C$	$t_M$	$x_0$	$x_L$	$x_C$	$x_M$	$k$
1.	3	7	17.5	26	0.03	0.20	1.44	1.84	0.126
2.	24	27	33	60	1.76	1.92	2.52	2.90	0.034

Diauxie can be observed when growth is plotted as function of the time of cultivation, as well as when the rate of growth is plotted against mycelial weight.

The characteristic kinetic constants of the above culture are given in Table 3.

Mycelial yields calculated with the above kinetic constants (determined graphically from Fig. 3) are marked on the growth curve by crosses.

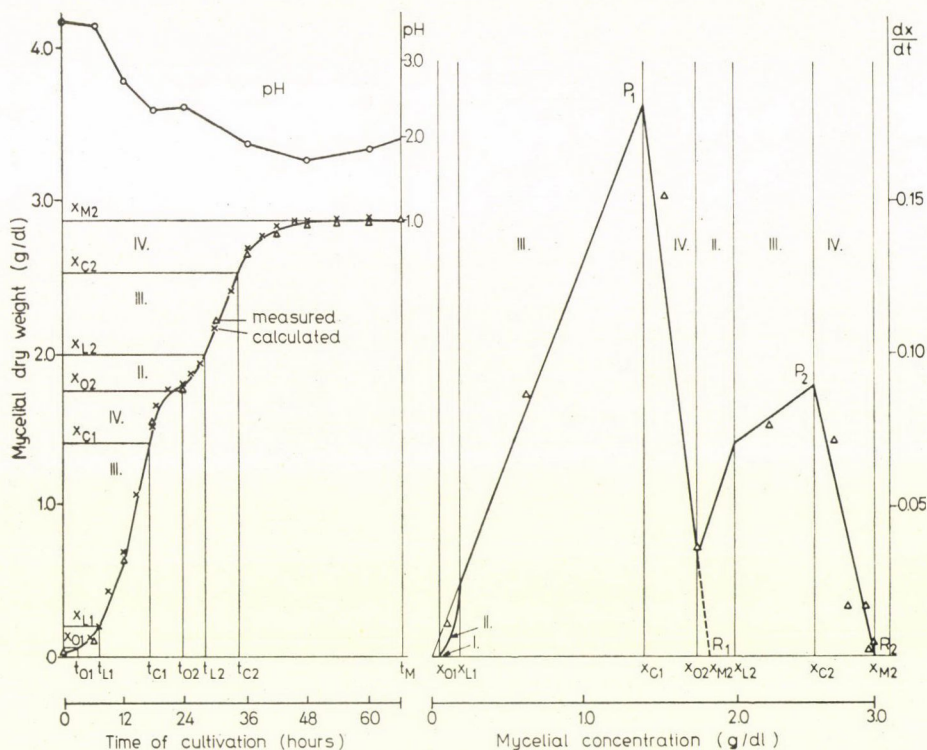


Fig. 3. The growth of *Aspergillus awamori* in medium No. 3 containing corn-steep-liquor and sucrose as the carbon source. Relationship between cell concentration and growth rate

Growth and rate of growth of *Aspergillus awamori* cultures in media containing sucrose and no corn-steep-liquor are depicted in Fig. 4.

As can be seen in the figure diauxic growth of *Aspergillus awamori* occurred also when cultivated in media containing sucrose as the carbon source without any corn-steep-liquor added.

The kinetic constants of the above cultivation are given in Table 4.



Table 4

Kinetic constants of *Aspergillus awamori* grown in media containing sucrose as carbon source and no corn-steep-liquor  
(Fig. 4)

Part of diauxie	$t_0$	$t_L$	$t_G$	$t_M$	$x_0$	$x_L$	$x_G$	$x_M$	$k$
1.	3	5.5	14.5	27.5	0.03	0.16	0.80	1.60	0.117
2.	24	27	34.5	60	1.30	1.50	2.30	2.50	0.054

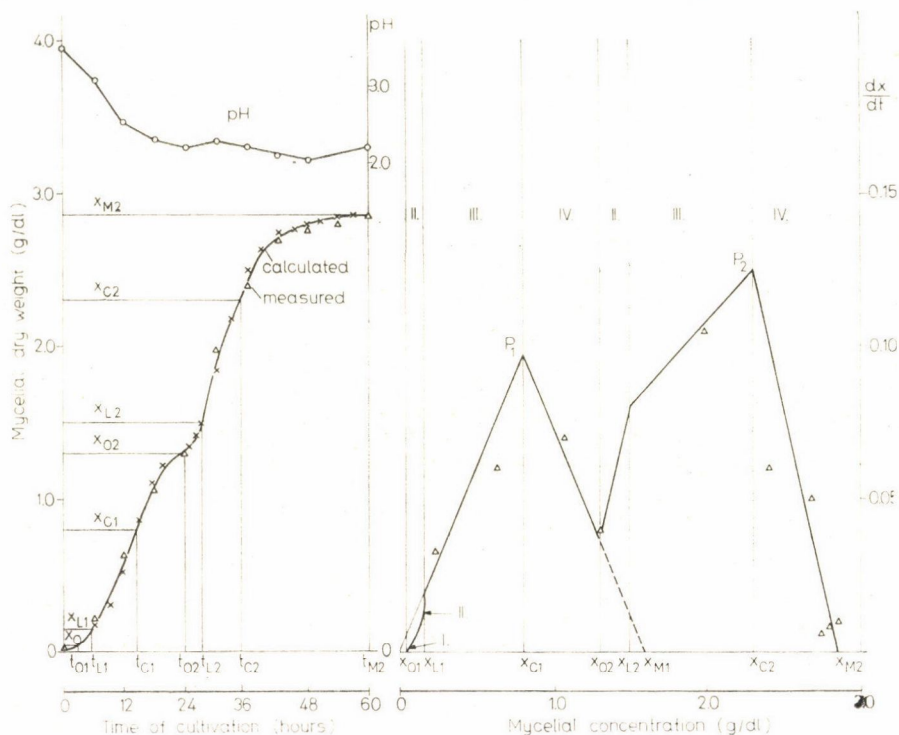


Fig. 4. The growth of *Aspergillus awamori* in medium No. 4 containing sucrose as the carbon source and no corn-steep-liquor. Relationship between cell concentration and growth rate

### 3. Conclusions

*Aspergillus awamori* strains showed diauxic growth in media containing pectin as carbon-source when corn-steep-liquor was present in the media (Fig. 1) and grew according to a simple four stage growth pattern, in media not containing corn-steep-liquor (Fig. 2).

In the first part of diauxic growth, assimilation of amino acids of corn-steep-liquor origin took place (Fig. 5) and monogalacturonic acid (hydrolysed by the enzymes of the culture) were assimilated only in the second part of the growth period (Fig. 6).

In cultures grown in pectin-containing media without corn-steep-liquor assimilation of monogalacturonic acid started in the first part of diauxic growth (Fig. 7).

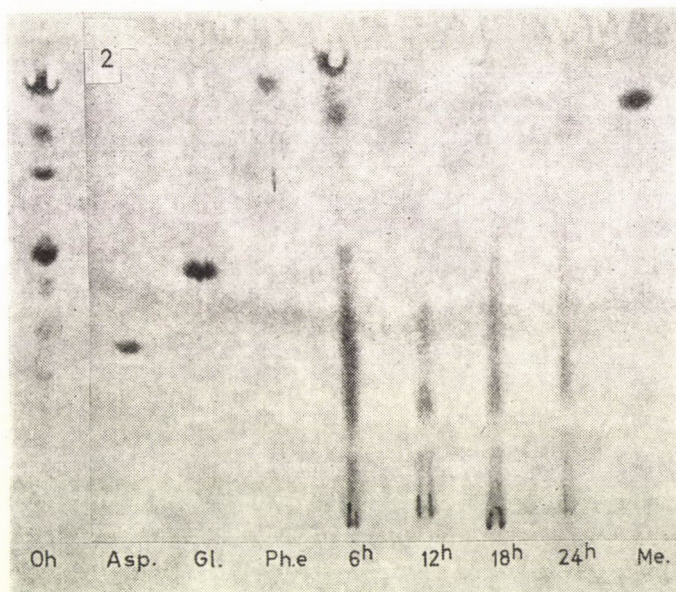


Fig. 5. Amino acid assimilation by *Aspergillus awamori* cultures in media containing corn-steep-liquor and pectin or sucrose as the carbon source (Media No. 1 and 3 resp.). Detection of amino acids in the fermentation broth as function of the time of cultivation, by thin-layer chromatography. (Spots: from left to right: O-hour, aspartic acid, glutamic acid, phenyl-alanine, 6-, 12-, 18-, and 24-hour fermentation broths and methionine.)

In the case of cultures grown in media containing sucrose as the carbon source and corn-steep-liquor, sugars (glucose and fructose) were assimilated mainly in the second part of diauxie (Fig. 8) while in media not containing corn-steep-liquor, assimilation of sugars commenced in the first part of the diauxic cycle (Fig. 9).

The reason for diauxie in this case is believed to be the difference in the utilisation of glucose and fructose produced by enzymic hydrolysis of sucrose. As can be seen in Fig. 9 glucose was first assimilated. This is in agreement with the results of FISCHER and co-workers (1951) who stated that  $\beta$ -h-fructosidase of the culture acts as transglucosidase liberating first



glucose from sucrose and building up an intermediate enzyme-fructose complex with the other component of the molecule.

\*

The authors are indebted to Ildikó GAJZÁGÓ for the preparation of amino acid chromatograms.

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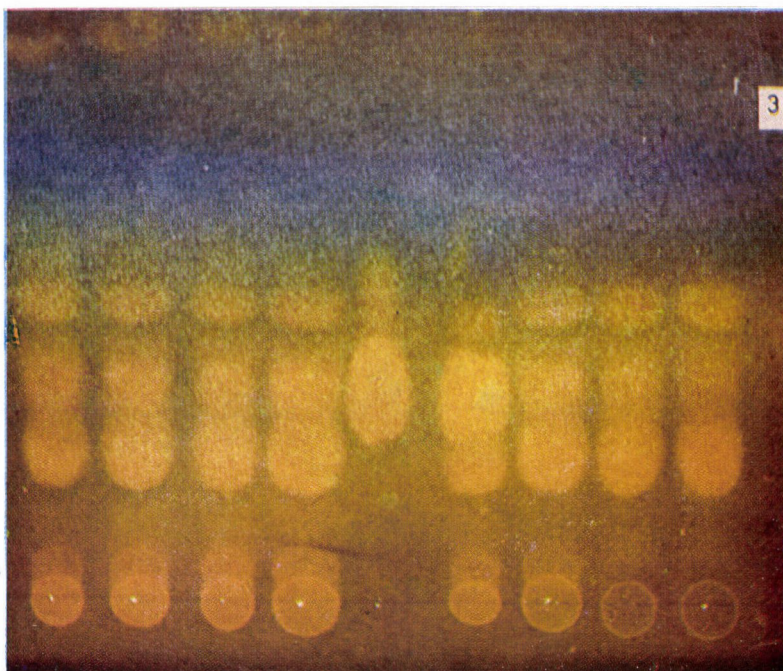


Fig. 6. Galacturonic acid assimilation by *Aspergillus awamori* in media containing corn-steep-liquor and pectin as the carbon source (Medium No. 1). Detection of mono-galacturonic acid and its oligomers in the fermentation broth by thin-layer chromatography (spots from left to right: 6-, 12-, 18-, 24-hr fermentation broths, monogalacturonic acid, 2 r sample + mono-galacturonic acid 30-, 36-, 42- and 4 r samples)

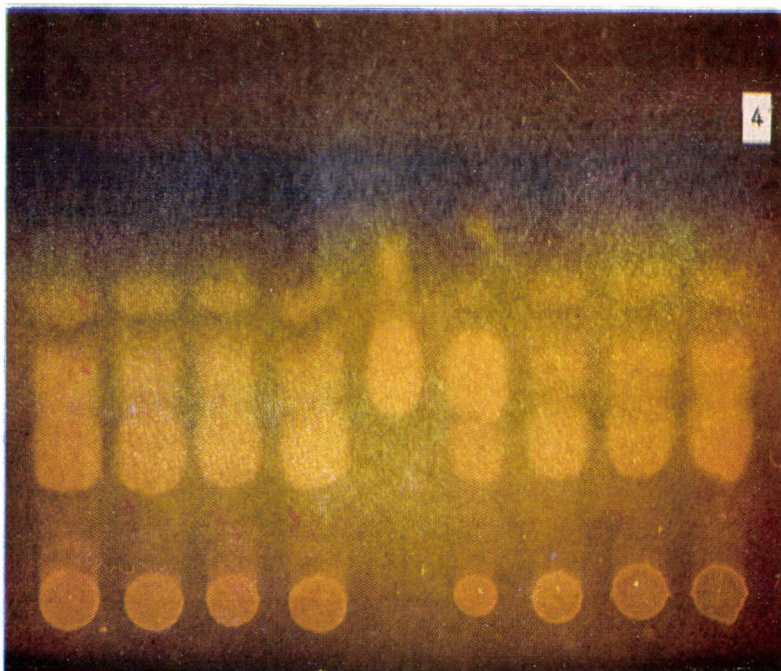


Fig. 7. Galacturonic acid assimilation by *Aspergillus awamori* in pectin-containing media, without corn-steep-liquor (Medium No. 2). Detection of amino acids by thin-layer chromatography (spots from left to right: 6-, 12-, 18-, 24-hr fermentation broth, mono-galacturonic acid, 24-hr sample + mono-galacturonic acid, 30-, 36-, 42- and 48-hr samples.)



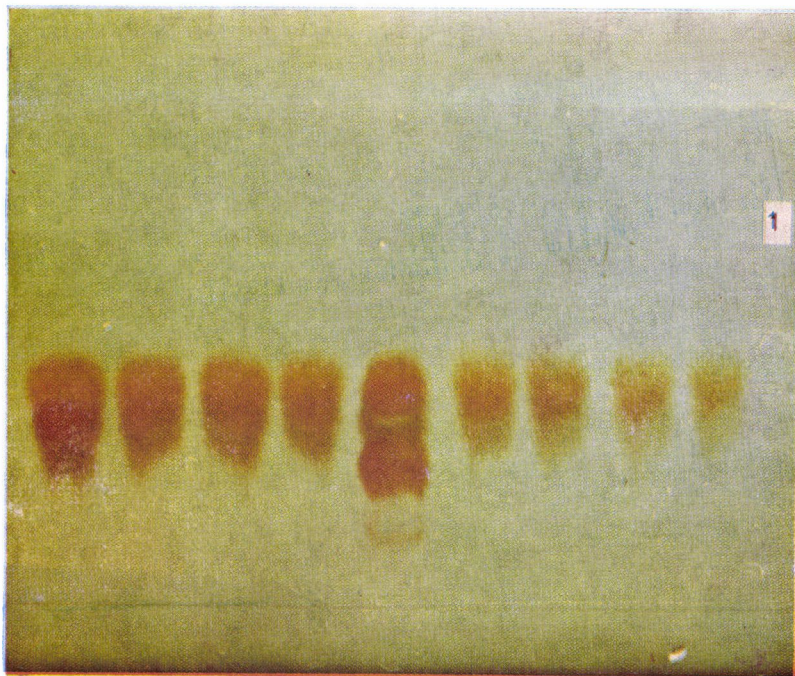


Fig. 8. The assimilation of sugars of *Aspergillus awamori* cultures in media containing corn-steep-liquor and sucrose as the carbon source (Medium No. 3). Detection of sugars in the fermentation broth by thin-layer chromatography (spots from left to right: 6-, 12-, 18-, 24-hr samples, test substances (fructose, glucose, sucrose), 30-, 36-, 42- and 48-hr samples)

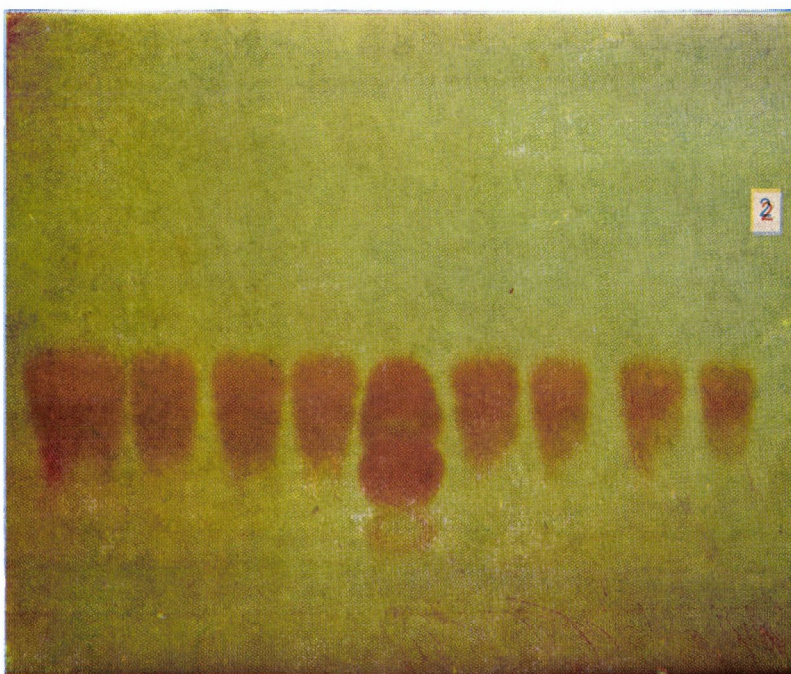


Fig. 9. The assimilation of sugars of *Aspergillus awamori* cultures in media containing sucrose as the carbon source and no corn-steep-liquor (Medium No. 4). Detection of sugars in the fermentation broth by thin-layer chromatography (spots from left to right: 6-, 12-, 18-, 24-hr samples, tests (fructose, glucose, sucrose), 30-, 36-, 42- and 38-hr samples)





## STUDY OF MILK CLOTTING ENZYMES

### VI. DISC ELECTROPHORESIS OF PREPARATIONS OF MICROBIAL AND ANIMAL ORIGIN

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The protein composition of 9, partly commercial and partly experimental milk clotting enzyme preparations of microbial origin, as well as of Rennin and Pepsin was studied by disc electrophoresis according to DAVIS (1964). The main results obtained are as follows:

1. The amount of protein necessary for electrophoresis is higher than that recommended for serum proteins due to poor staining with Amido Black of the substances investigated.

2. The number of protein fractions obtained by electrophoresis of the different preparations ranged from 2 to 9.

3. The electrophoretic patterns including relative mobilities, widths and colour intensities of the protein zones are suitable to characterize the individual preparations.

4. Two distinct, fast moving protein bands appear in the electrophoretograms of all milk clotting preparations tested.

5. Inhomogeneity or excessive salt content of the preparations may affect both the relative mobilities and the number of protein zones in the electrophoretograms.

6. Desalting by gel filtration (Figs. 2, 3 and 5) may increase or decrease (Fig. 6) the number of the protein zones obtained on subsequent electrophoresis.

7. Disc electrophoresis proved suitable to follow the purification of the milk clotting enzyme preparation E 31 (see the electrophoretograms E 31 and E 311).

8. In contrast to pH 6.0, TRIS buffer at pH 8.3, used in this method inactivates both the milk clotting and the proteolytic activities of most of the preparations, thus no information on the enzymic character of the individual protein bands of the electrophoretograms could be obtained.

In a previous paper (VAMOS & MORVAI-RÁCZ, 1970) an account was given on the paper electrophoretic study of 2 commercial calf rennin and 4 fungal milk clotting preparations. Using buffers of different composition, ionic strength and pH, up to 3 protein zones of different mobilities and — in some cases — of different signs of charge could be obtained. Since all the preparations tested were crude products and thus probably contained several protein constituents, it was assumed that a more complete separation of the latter could be achieved by polyacrylamide gel disc electrophoresis.

The answer was sought to the following questions:

1. whether disc electrophoresis was suitable to characterize commercial rennins without previous purification,

2. whether there was a possibility to differentiate between preparations of animal and microbial origin on the basis of their disc electrophoretic pattern,



3. whether the milk clotting and proteolytic activities of the preparations could be separated from each other by this technique and finally,

4. whether the purification of an enzyme preparation could be followed by disc electrophoresis.

## 1. Materials and methods

### 1.1. *The enzyme preparations tested and some of their characteristics*

The names, the producer or trading firms and the origin of the 11 preparations tested are given in Table 1 along with some of their characteristics important from the point of view of disc electrophoresis, such as moisture and protein content, inorganic matter and molecular weight. The latter values were taken from literature, the corresponding sources are given in the table.

**1.1.1. Moisture content and inorganic matter.** Both characteristics were determined by differential thermal analysis, using a PAULIK, PAULIK & ERDEY type Derivatograph, Model MOM, Budapest.

The weight of the samples analysed ranged from 50 to 120 mg, heating was carried on for 100 min till the temperature inside the sample reached 900 °C. Moisture content was calculated from the corresponding loss of weight and inorganic matter from the residue, as could be read from the TG curve. Both quantities were expressed as percentage of the crude preparations.

**1.1.2. Protein content.** Protein content was estimated by UV spectrophotometry using solutions of various concentrations of the preparations. The protein content was calculated from readings taken at 280 and 260 nm, according to WARBURG and CHRISTIAN (1941).

**1.1.3. Measurement of enzyme activities.** Milk clotting and proteolytic activities were assessed according to the methods developed earlier in this laboratory (VÁMOS *et al.*, 1969; VÁMOS & MORVAI, 1970). Since the biuret reaction could not be carried out in TRIS buffer, because of its complexing effect on copper ions, a slight modification was introduced, i.e. proteolytic activity was determined spectrophotometrically from readings at 280 nm of trichloroacetic acid soluble matter.

The effect of TRIS buffer on enzyme activity was established by adding 1 ml of 0.5% w/v enzyme solution to 4 ml buffer (pH 8.3 and 6.0, respectively) and incubating the mixture for 2 h at room temperature. Activities were measured immediately after incubation and expressed as percentage of the activities determined in enzyme solutions incubated in 0.1 M pH 6.0 phosphate buffer (DAWSON *et al.*, 1959). Previous experience had shown that no losses of either activity occurred on storage in this buffer (VÁMOS & MORVAI, 1970).

Table 1  
The enzyme preparations tested and some of their characteristics

Name or sign of preparation	Manufacturers or trading company	Origin of the preparations	Characteristics of the preparations			
			Moisture content, %**	Protein content, %***	Inorganic matter, %***	Molecular weight · 10 <sup>-3</sup> +
Meito Rennet	Takasago Perfumery Co., Tokyo, Japan	Mold, surface culture	5	44.3	17	26.8—30.6 (YU <i>et al.</i> , 1968)
Rennilase	Novo Industri A/S Denmark	Mold, submerged culture	—	17.4 <sup>++</sup>	—	—
Sure Curd	Chas. Pfizer & Co., Inc., New York, N.Y., USA	Mold, submerged culture	5	27.3	62	34—39 (SARDINAS, 1968)
E 31	KÉKI*, laboratory preparation	Mold, surface culture	13	37.9	15	—
E 311	KÉKI*, laboratory preparation	Mold, surface culture	11	—	3	—
E 32	KÉKI*, laboratory preparation	Mold, submerged culture	14	37.0	15	—
E 41	KÉKI*, laboratory preparation	Mold, surface culture	20	20.0	23	—
E 42	KÉKI*, laboratory preparation	Mold, submerged culture	14.8	28.3	0	—
Neutral protease	Chinoin Pharmaceutical and Chemical Factory, Budapest, Hungary	Bacterial, submerged culture	5	54.9	40	25—30 <sup>+++</sup> (BOYER <i>et al.</i> , 1960) 26.7 <sup>++++</sup> (BARMAN, 1969)
Pepsin	Stockpiling Company for Pharmaceuticals, Budapest	Animal (swine stomach)	20	2.7	0	36.0 (BARMAN, 1969) 27—35 (BOYER <i>et al.</i> , 1960)
"Clotting powder"	Stockpiling and Wrapping Manufacturers Company for the Meat Industry, Budapest	Animal (calf stomach)	1	1.3	85.7	34; 30.7 (BARMAN, 1969)
Rennin	Nutritional Biochemicals Company, Cleveland, Ohio, USA	Animal (calf stomach)	0.4	4.0	95.6	34; 30.7 (BARMAN, 1969)

\* = KÉKI: Central Food Research Institute, Budapest, Hungary

\*\* Determined with the Derivatograph from samples of 50—120 mg, heating up to 900 °C in 100 min.

\*\*\* Determined spectrophotometrically from absorbances at 260 and 280 nm (WARBURG & CHRISTIAN, 1941)

+ From literature data

<sup>++</sup> w/v

<sup>+++</sup> Alkaline protease from *B. subtilis*

<sup>++++</sup> Subtilopeptidase A (Subtilisin)



## 1.2. The electrophoretic run

*1.2.1. Preparation of the gel.* A 7.5% basic polyacrylamide gel was prepared according to DAVIS (1964) in 5 mm  $\varnothing$  glass tubes of 100 mm length. Instead of the sample gel 0.20 ml 40% w/v sucrose solution was layered on the bottom of the tubes fixed upside down in the respective moulds and followed by the spacer and the small-pore gels. The sample solutions were pipetted on top of the former, after the tubes had been put in their final position into the electrophoretic apparatus.

The pH of all the buffers used, including the TRIS — glycine electrode buffer (pH 8.3) was checked in a Metrohm E 166 type pH-meter.

*1.2.2. Electrophoretic equipment and separation.* The gel electrophoresis apparatus of the firm *Reanal*, Budapest, fitted with carbon electrodes and allowing the simultaneous run of 12 tubes, was used. The apparatus was connected with a 60 mA (300 V) power supply manufactured by MIM Műszeripari Művek, Budapest-Esztergom.

The necessary amounts of the individual preparations had been determined in preliminary runs and ranged from 0.5 to 9.4 mg protein per gel. These quantities were layered in volumes of 0.05 to 0.10 ml (made up with distilled water) on top of the spacer gel. In order to follow the electrophoretic run 0.05 ml of 0.5% w/v bromophenol blue dissolved in 1% aqueous acetic acid was added to 100 ml of the upper (cathode) buffer.

Direct current of 5 mA/tube was applied and electrophoresis continued at room temperature for 2.5–3 h. During this time the downward buffer flow travelled about 5 to 6 cm as indicated by the bromophenol blue band. After the run the position of this band was marked by a needle scratch on the extruded gels.

*1.2.3. Staining and evaluation of the protein zones.* The extruded gels were transferred into test tubes and kept for 1 h in the staining solution (1 g Amido Black dissolved in 100 ml of 7% acetic acid). Destaining was carried out in the same tubes by changing the 7% acetic acid destaining solution several times until the background between the protein zones became colourless.

The distances travelled by the protein zones as well as by bromophenol blue were measured with a ruler and mobilities related to the latter ( $M_B$ ) were calculated. The width of the zones was measured in a similar way and expressed in mm.

Generally the same enzyme preparation was run in all the 12 tubes to ensure the symmetry of the electric field, since the solutions of the various crude and commercial enzyme preparations were expected to have very different conductivities due to the large amounts of carrier or stabilizing electrolytes (e.g. NaCl) which some of them contained. Some runs have been carried out in triplicate.

Reproducibility of the results was examined by analysis of variance and Student's *t*-test (SvÁB, 1967).

### *1.3. Desalting of commercial preparations*

Some commercial preparations, such as calf stomach rennin, with extremely high sodium chloride and low protein content, were desalted by gel filtration prior to the electrophoretic separation. Gel filtration was carried out on Sephadex G 25 or G 10 gel beds of 2 cm  $\varnothing$  and 30 cm height. Column loads ranged from 0.33 to 2.2 ml enzyme solution containing 14–22 mg of protein.

Elution was carried out with distilled water at a rate of 1.6–1.8 ml/min, 5 ml fractions were collected using a "Labor" type fraction collector. Protein content was determined in all the fractions and enzyme activities measured in those containing protein. All fractions were tested qualitatively for chloride with a 1% w/v silver nitrate solution.

The active fractions from 2 or 3 parallel gel filtration runs were combined and concentrated at 40 °C in a rotational vacuum evaporator "Rotadest" to a volume of 2–3 ml. The protein content of the concentrated solution was determined and recovery calculated.

Aliquots of the concentrated solutions corresponding to 0.5–2.6 mg protein per gel were taken for gel electrophoresis.

## **2. Results**

### *2.1. Effect of TRIS buffer on enzyme activity*

The milk-clotting and proteolytic activities measured after a 2-h incubation of the enzyme preparations in TRIS buffer of pH 8.3 and 6.0 resp., are summarized in Table 2.

As can be seen, in TRIS buffer, pH 8.3, eight out of eleven preparations were completely inactivated with respect to both milk clotting and proteolytic activities, in two cases the activities were reduced (E 32 and Neutral protease), whereas with Rennilase a strong stimulating effect could be noted. The same buffer adjusted to pH 6.0 increased the activities of the majority of the preparations.

### *2.2. The disc electrophoretic pattern of the preparations tested*

*2.2.1. Microbial preparations.* The relative mobilities ( $M_B$ ) of the protein zones obtained on disc electrophoresis of the microbial preparations examined as well as the distance travelled by the bromophenol blue marker are given in Table 3.



Table 2

*The effect of tris-buffer on the milk-clotting and proteolytic activities of the enzyme preparations*  
 Tris-buffers containing 0.1% w/v enzyme preparation; incubation: 2 h; residual activities expressed as percentage of the values measured (for proteolytic activity by the absorbance method), after incubation in 0.1 M phosphate-buffer, pH 6.0 under similar conditions

Name or sign of preparation	Activities in 0.1 M pH 6.0 phosphate buffer (DAWSON <i>et al.</i> , 1959)			Residual activities in 0.05 M tris-buffer (DAWSON <i>et al.</i> , 1959)			
	Milk-clotting SU/g*	Proteolytic U/g**		pH 8.3		pH 6.0	
		Biuret-reaction	Absorbance at 280 nm	Milk-clotting, %	Proteolytic, %	Milk-clotting, %	Proteolytic, %
Meito Rennet	228 000	6 550	3 300	0	0	70	106
Rennilase	6 400	460	420	208	262	175	83
Sure Curd	114 000	7 600	3 500	0	0	233	137
E 31	61 000	6 300	2 500	0	0	77	92
E 32	66 000	12 500	7 100	87	86	111	63
E 41	42 000	5 500	3 400	0	0	157	124
E 42	47 000	4 850	2 100	0	0	283	126
Neutral protease	250 000	15 500	13 000	32	28	160	42
Pepsin	28 000	510	440	0	0	62	461
"Clotting powder"	47 000	630	550	0	0	121	120
Rennin	66 000	620	370	0	0	202	268

\* 1 Soxhlet Unit (SU) = the amount of milk in ml, coagulated in 40 min by 1 g of enzyme preparation at 35 °C;

\*\* 1 Unit (U) = the amount of casein (in mg) decomposed in 1 h by 1 g of enzyme at 35 °C and pH 6.0.

Table 3  
*Relative mobilities of the protein zones of*  
 Conditions of electrophoresis: 2.5 h,

Name or sign of preparation	Protein mg per gel	Number of parallel gel tubes	Number of protein zones obtained	Mobilities of the zones related to							
				10—20		21—30		31—40		41—50	
				$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Meito Rennet	6.5	5	4	—	—	—	—	—	—	—	—
Rennilase	8.7	11	2	—	—	—	—	—	—	—	—
Sure Curd	6.5	11	3	—	—	—	—	—	—	—	—
E 31	9.4	10	3	—	—	—	—	—	—	—	—
E 311	—*	12	2	—	—	—	—	—	—	—	—
E 32	5.8	12	5	—	—	—	—	—	—	40	4.6
E 41	1.3	11	3	—	—	—	—	—	—	—	—
E 42	2.8	11	3	14	1.8	—	—	—	—	—	—
Neutral protease	5.7	12	3	—	—	—	—	—	—	—	—

\* 0.07 ml per tube of a 10% w/v enzyme solution (~ 7 mg enzyme preparation per tube)

As can be seen, the number of the protein zones obtained on disc electrophoresis ranged from 2 to 5. All patterns had two bands in the relative mobility range of 90 to 100, whereas values below 70 were rare.

The variation coefficients of the  $M_B \cdot 100$  values are much lower than those of the distances travelled by bromophenol blue. That means that, in spite of considerable variations in the latter, the electrophoretic pattern is fairly reproducible. This has been confirmed by analysis of variance and Student's *t*-test for one of the preparations (E31) chosen at random: three enzyme solutions of identical concentration (32% w/v) were prepared and amounts ranging from 9.4 to 9.8 mg protein per tube were taken for disc electrophoresis. Two of the solutions were run once in twelve tubes each and one solution was run on 3 instances in a total of 36 tubes. The relative mobilities obtained in the five runs were compared. No significant differences were obtained between the corresponding  $M_B \cdot 100$  values on applying the same enzyme solution in different runs. Significant differences were noted in two out of 5 cases on comparing the electrophoretic patterns of the different enzyme solutions.

The amounts of protein applied per tube as indicated in Table 3 exceed the values recommended in the literature (DAVIS, 1964; MAURER, 1968) by one order of magnitude. This is due to the fact that most of the preparations give a very poor stain with Amido Black.

The disc electrophoretic patterns of the preparations are illustrated in Fig. 1, where, beside the  $M_B$ -values, the spreading and colour intensity of the zones is taken into consideration as well.

*microbial milk-clotting enzyme preparations*

5 mA/gel, room temperature

bromophenol blue ( $M_B$ ) $\times$ 100												Distance travelled by bromophenol blue, cm	
51—60		61—70		71—80		81—90		91—95		96—100			
$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s		
—	—	—	—	71	5.0	80	5.0	93	1.0	99	0.0	6.3	0.33
—	—	—	—	—	—	—	—	91	3.0	96	3.0	5.5	0.42
—	—	—	—	71	0.9	—	—	93	0.5	98	0.3	6.4	0.11
—	—	—	—	—	—	83	3.0	92	3.0	97	6.0	5.7	0.78
—	—	—	—	—	—	—	—	90	1.0	98	1.0	5.0	0.49
50	6.0	—	—	—	—	86	1.8	93	1.4	97	0.8	5.5	0.37
—	—	60	4.0	—	—	—	—	91	1.0	96	0.7	4.9	0.43
—	—	—	—	—	—	—	—	93	1.0	98	0.5	5.4	0.43
—	—	—	—	73	3.0	—	—	93	0.7	98	0.6	5.9	0.40

$\bar{x}$  = mean

s = standard deviation



The protein bands of the different preparations stain differently with Amido Black. The fractions of E 32 are, *e.g.*, much fainter than those of E 41, although the amount of protein applied per tube was more than 4 times as high with the former as with the latter preparation (Table 3).

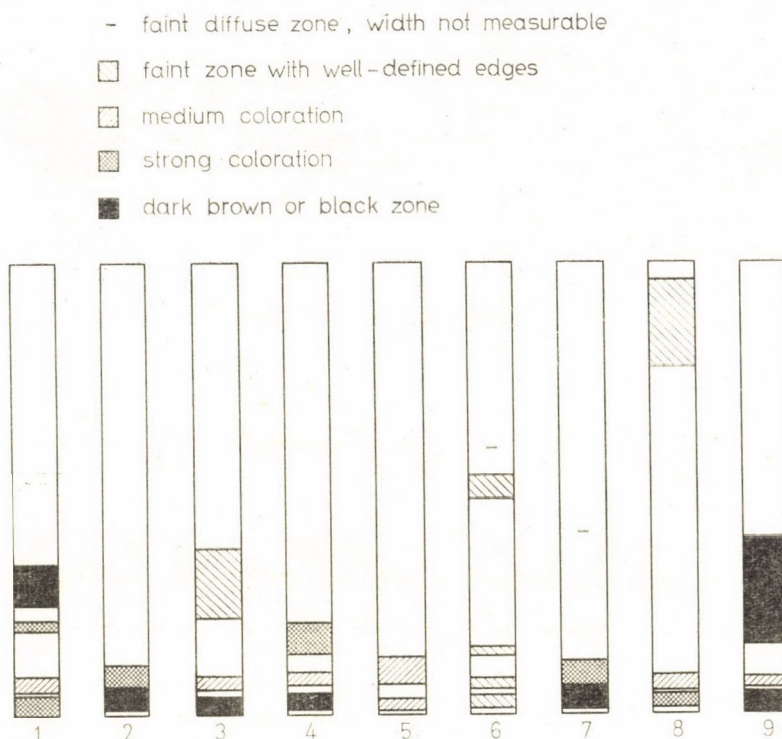


Fig. 1. Disc electrophoretic protein patterns of microbial rennin preparations. Gel and buffer systems according to DAVIS (1964). Conditions of electrophoresis: 2.5 h, 5 mA/gel, room temperature. Preparations: 1. Meito Rennet; 2. Rennilase; 3. Sure Curd; 4. E 31; 5. E 311; 6. E 32; 7. E 41; 8. E 42; 9. Neutral protease

The zones of the lowest  $M_B$ -values of the preparations E 32 and E 41, respectively, are very faint and diffuse, with ill-defined edges and are, therefore, marked on the figure only by a dot indicating their approximate center.

**2.2.2. Preparations of animal origin.** The disc electrophoretic resolution of this group of proteins presented far more difficulties than that of the microbial preparations. Only pepsin gave a reproducible pattern when applied without pretreatment, whereas solutions of the calf stomach preparations yielded alternately 3 or 4 fractions, some of which differed significantly with respect to their relative mobilities. It was assumed that this phenomenon was due to the high electrolyte (NaCl), or low protein content, resp., and the resulting inhomogeneity of the preparations. Desalting by gel filtration was, therefore, thought necessary prior to electrophoresis.

2.2.2.1. *Desalting of calf rennin.* — The gel filtration elution patterns obtained on Sephadex G 25 of the "Clotting powder" and of "Rennin" are represented in Figs. 2 and 3.

Column loads were 2 and 2.2 ml, resp., corresponding to 14.8 mg of protein for "Clotting powder" and 17.9 mg for "Rennin." The first as well as

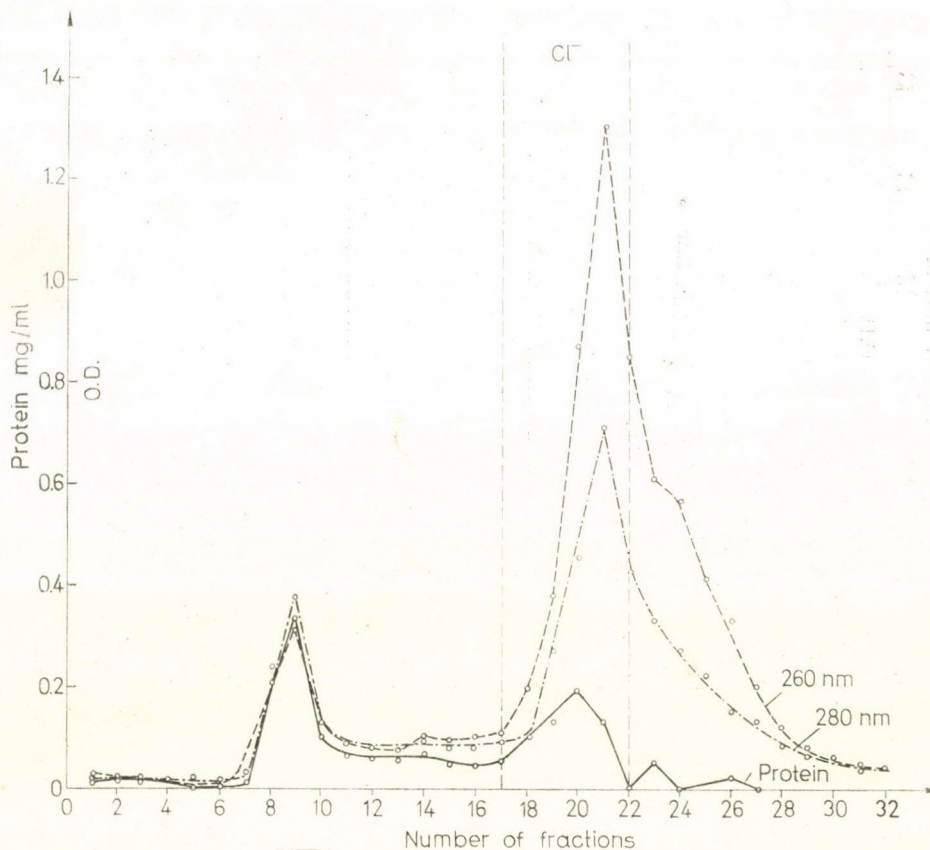


Fig. 2. Desalting of "Clotting powder" by gel filtration on Sephadex G 25. Column load: 2 ml, 80% w/v filtered enzyme solution containing 14.8 mg protein. Elution: distilled water, 1.6 ml/min. Protein recovery: 8.0 mg (54%). Ratio of chloride free and chloride containing "protein": (63.4 : 36.6)

the last chloride containing fraction were both marked by dotted lines drawn in parallel with the ordinate. It can be seen that with both enzymes the main protein peak as calculated from the absorbances at 260 and 280 nm was eluted before the salt. A second "protein" peak was obtained together with sodium chloride. This latter is an artefact containing low molecular impurities, mainly colouring matter, with a strong absorbance at 260 nm. (WARBURG & CHRISTIAN's formula is only valid for proteins containing less than 20% non-protein



impurities.) Consequently, neither milk clotting, nor proteolytic activity could be detected in the respective fractions, therefore they were discarded. The apparent protein content of the second peak was taken into account in calculating protein recovery after gel filtration, because its impurities were present in the starting solution and influenced the value obtained as protein from the absorbance data. The protein ratio in the two peaks was 63.4 : 36.6

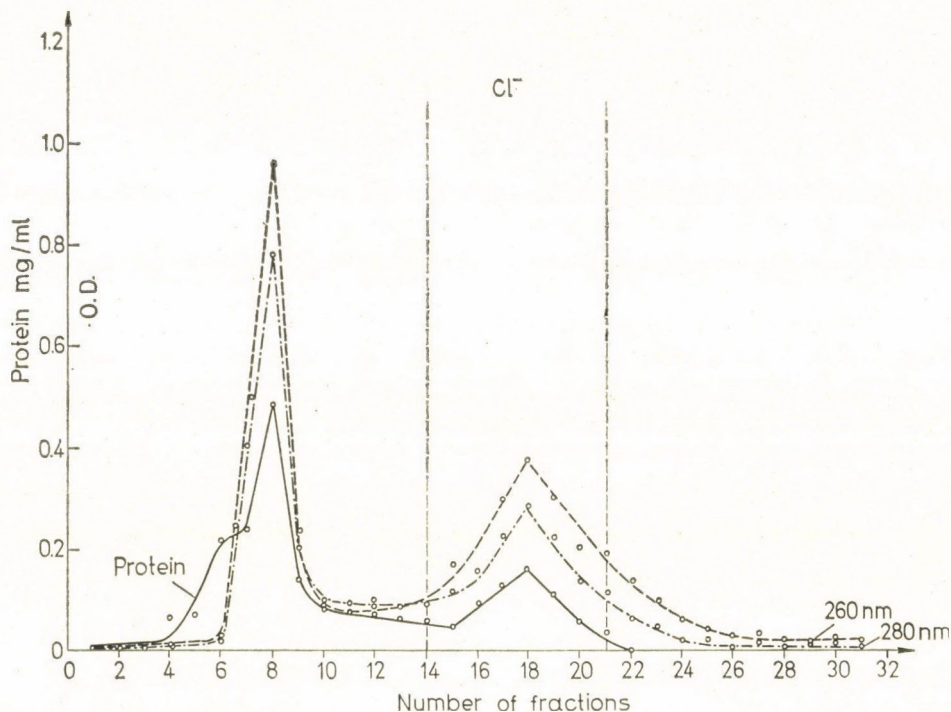


Fig. 3. Desalting of "Rennin" by gel filtration on Sephadex G 25. Column load: 2.2 ml, 25% w/v filtered enzyme solution containing 17.9 mg protein. Elution: distilled water, 1.6 ml/min. Protein recovery: 11.5 mg (64%). Ratio of chloride free and chloride containing "protein": (68.9 : 31.1)

for "Clotting powder" and fairly similar for "Rennin" (68.9 : 31.1). Protein recovery was rather poor, 54% in the first and 64% in the second case. This might be primarily due to the impurities mentioned above, but partly also to inaccuracy in diluting the fractions with optical densities above 0.8.

The elution pattern with Sephadex G 10 was essentially the same, although the separation of the two peaks was less distinct.

*2.2.2.2. Disc electrophoresis of commercial and desalted preparations.* — The distances travelled by bromophenol blue as well as the relative mobilities in disc electrophoresis of the commercial and desalted milk-clotting enzyme preparations of animal origin are presented in Table 4.

The data in the table indicate that of all the preparations tested pepsin gave the highest number of fractions, i.e. 9. The commercial preparations of "Clotting powder" and "Rennin" both manufactured from calf stomach, bore some resemblance in their electrophoretic patterns, although the relative mobilities of the slowest fractions differed highly significantly from each other.

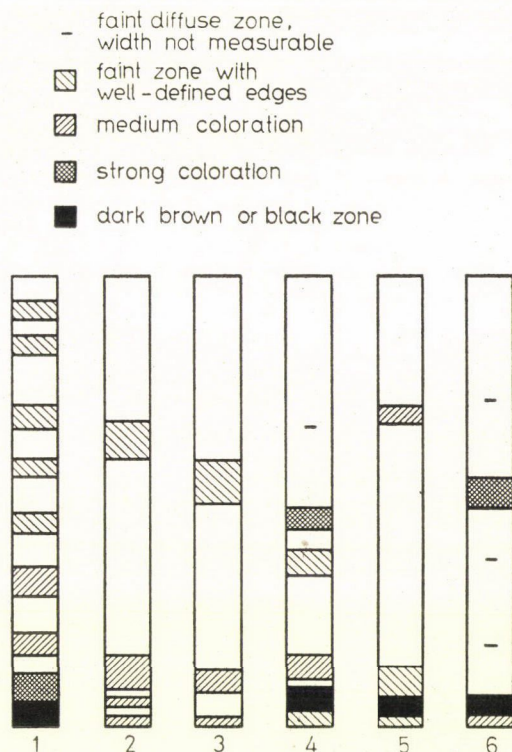


Fig. 4. Disc electrophoretic protein patterns of milk clotting preparations of animal origin. Gel and buffer systems according to DAVIS (1964). Conditions of electrophoresis: 2.5 h, 5 mA/gel, room temperature. Preparations: 1. Pepsin; 2. "Clotting powder", commercial, I; 3. "Clotting powder", commercial, II; 4. "Clotting powder", desalted; 5. "Rennin", commercial; 6. "Rennin", desalted

This applies also to the patterns of "Clotting powder" obtained from two different solutions. In this case the difference was even more striking, since one of the solutions yielded 4, while the other one, of higher concentration, only 3 fractions.

Desalting has a considerable effect on the electrophoretic pattern of both calf rennet preparations, it increases the number of the protein bands obtained to 6. The location of the zones is similar for the two desalted preparations, although the relative mobilities of three corresponding fractions differ highly significantly from each other. It must be noted, however, that



these three bands are faint and thus their relative mobilities could be established with less accuracy. Fig. 4 gives an idea of the electrophoretic patterns of this latter group of enzyme preparations.

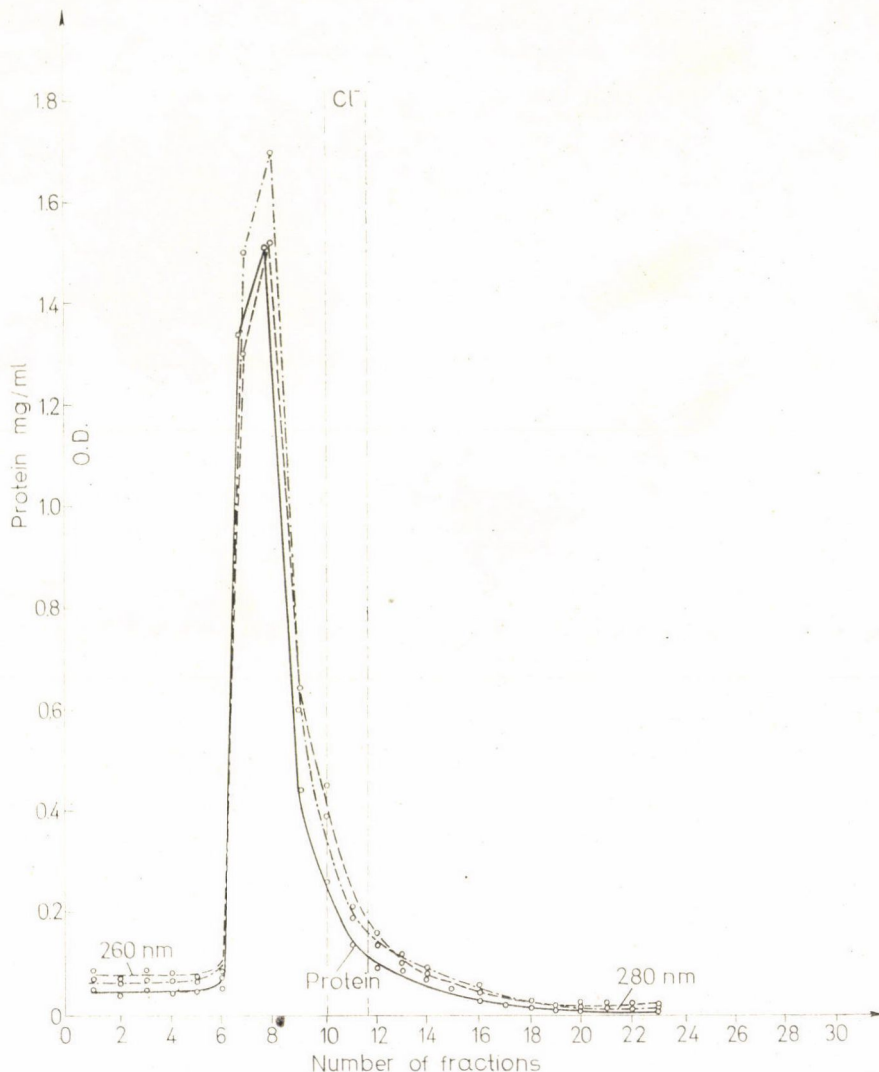


Fig. 5. Desalting of Meito Rennet by gel filtration on Sephadex G 10. Column load: 0.33 ml, 15% w/v filtered enzyme solution containing 22 mg protein. Elution: distilled water, 18 ml/min. Protein recovery: 21.86 mg (99.4%). Ratio of chloride free and chloride containing "protein": (88.8 : 11.2)

*2.2.3. The effect of desalting on the electrophoretic behaviour of Meito Rennet.*  
In order to check the effect of desalting on the gel electrophoretic migration of the protein bands a salt containing microbial rennin, Meito Rennet, which

had yielded a reproducible pattern in the commercial form (Table 1), was subjected to gel filtration. The respective elution pattern is illustrated in Fig. 5.

As can be seen, only one protein peak was obtained in this case and chloride was found in 3 fractions only. The weight ratio of chloride free and chloride containing protein was 88.8 : 11.2 and protein recovery 99.4%.

The electrophoretic pattern of desalted Meito Rennet showed some deviation from that of the commercial preparation, as can be seen in Fig. 6.

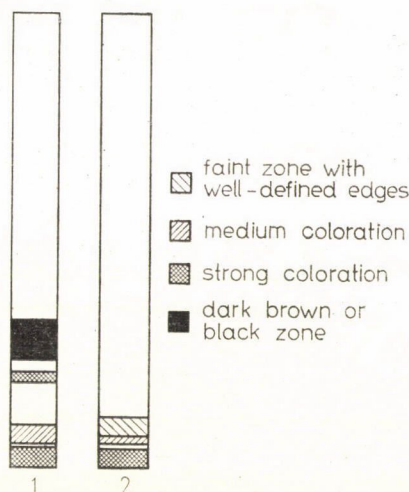


Fig. 6. Effect of desalting on the electrophoretic protein pattern of Meito Rennet. Gel and buffer systems according to DAVIS (1964). Conditions of electrophoresis: 2.5 h, 5 mA/gel, room temperature. Preparations: 1. Meito Rennet, commercial; 2. Meito Rennet, desalted

The slowest protein zone of the commercial preparation is missing from the electrophoretogram of the salt free solution and the next band is much fainter and broader in the latter besides being shifted towards the third one. These deviations cannot be explained by the differences in the amounts of protein applied (6.5 mg and 2.5 mg per tube of the commercial and the salt free preparation, respectively), as in the preliminary runs carried out to establish the optimal quantities for electrophoresis 1.9 mg per tube of the former still yielded 4 well-defined bands. The relative mobilities of the three protein zones which appear on the electrophoretogram of the salt free fraction, are  $90 \pm 1.4$ ,  $94 \pm 0.1$  and  $98 \pm 0.3$ , the two latter being practically identical with the corresponding values found for the commercial preparation (Table 3).



### 3. Conclusions

Not all the questions put in the introduction can be answered satisfactorily by the results obtained so far.

It can be said that commercial milk clotting enzyme preparations of both microbial and animal origin may be characterized by the protein pattern obtained on disc electrophoretic separation according to DAVIS (1964). If the electrolyte (salt) content of the preparation related to its protein content is not too high, no desalting is needed prior to electrophoresis. A salt to protein ratio of about 2.3, as in Sure Curd, still allows to obtain reproducible protein patterns. Non-electrolyte enzyme stabilizers or carriers, as lactose in Pepsin, do not interfere at all with the electrophoretic separation.

All preparations investigated gave two distinct fast moving protein bands which did not disappear on desalting and remained unchanged even after different steps of purification (E 311 is a purified form of E 31, obtained by fractionated precipitation and ion-exchange chromatography). The slower moving bands are, for the different preparations, more or less different in relative mobility, width and colour intensity and thus suitable to characterize the individual enzymes. Strictly identical experimental conditions are, of course, required to obtain reproducible protein patterns. Since the relative mobilities, particularly of the slower moving characteristic zones may differ significantly when obtained from different solutions of similar concentration of the same preparation, as has been proved by analysis of variance, it is

Table 4

*Relative mobilities of the protein zones of milk*  
Conditions of electrophoresis:

Name or sign of preparation	Protein mg per gel	Number of parallel gel tubes	Number of protein zones obtained	Mobilities of the zones related to							
				0—10		11—20		21—30		31—40	
				$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Pepsin	2.0	9	9	0.8	0.1	16	1.0	—	—	32	2.0
"Clotting powder", commercial, I*	0.5	18	4	—	—	—	—	—	—	37	2.0
"Clotting powder", commercial, II*	1.3	14	3	—	—	—	—	—	—	—	—
"Clotting powder", desalted	0.5	9	6	—	—	—	—	—	—	34	2.9
Rennin, commercial	1.0	9	4	—	—	—	—	—	—	31	2.5
Rennin desalted	0.7	11	6	—	—	—	—	28	2.2	—	—

\* The mobilities given are mean values of two electrophoretic runs carried out with the same enzyme solution; I and II are different solutions of the same preparation

advisable to consider not only these values but also the protein pattern, as an entity, including breadth and colour intensity (even shade) of the bands. Thus, e.g., Rennilase and E 311 have both only two protein bands of the same relative mobilities in their electrophoretic patterns (Table 3). This, however, as illustrated in Fig. 1 allows a clear distinction of the two preparations on the basis of the width (and colour intensity) of the bands: with Rennilase, the two bands are quite close to each other and can be distinguished only by their colour shade: the faster moving band is black, while the other one is of a medium brown. With E 311 the two bands are separated by a clear gel zone, due to the fact that the faster band is very sharp and narrow.

It is perhaps interesting to note that the colours of the bands of all the preparations range from light brown to black with the exception of Pepsin which gave a blue-green stain with Amido Black.

Since both the milk clotting and the proteolytic activities of the great majority of the preparations were completely inactivated in TRIS buffer, pH 8.3 (Table 2) no attempt was made at testing the electrophoretically separated proteins for enzyme activity. This deficiency might be overcome by altering both gel and electrode buffers.

Increasing the gel concentration is another alteration of the gel system which seems indicated as the molecular weights of the commercial preparations tested are in the range of 30 000 (Table 1), i.e. much lower than those of the serum proteins, for the separation of which the method had been originally developed (DAVIS, 1964).

*clotting enzyme preparations of animal origin*  
2.5 h, 5 mA/gel, room temperature

bromophenol blue ( $M_B$ ) $\times$ 100														Distance travelled by bromophenol blue, cm	
41—50		51—60		61—70		71—80		81—90		91—95		96—100			
$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
43	2.0	55	2.0	68	2.0	—	—	82	8.0	91	1.2	98	0.1	5.4	0.47
—	—	—	—	—	—	—	—	88	1.6	95	1.1	100	0.0	5.9	0.78
46	2.2	—	—	—	—	—	—	—	—	95	1.0	100	0.0	4.5	0.30
—	—	54	3.0	64	2.6	—	—	87	1.6	94	1.6	100	0.0	4.1	0.28
—	—	—	—	—	—	—	—	90	1.2	95	1.2	100	0.0	5.0	0.47
48	2.1	—	—	63	2.2	—	—	82	3.7	95	0.1	100	0.0	4.0	0.30

$\bar{x}$  = mean

s = standard deviation



Purification of any kind changes the electrophoretic pattern. Desalting increased the number of the protein bands of both calf stomach rennin preparations. Although the relative mobilities of 3 out of the 6 zones of the two desalted rennins were significantly different (Table 4), the electrophoretograms were essentially similar as were those of the commercial preparations with 4 bands each (Fig. 4).

Desalting had an opposite effect on Meito Rennet in that it decreased the number of the protein zones (although protein recovery in gel filtration was by far the best of all with this enzyme). This indicated that desalting may produce various effects which may be due to different causes (e.g. association or dissociation of proteins). It is advisable, therefore, when comparing different preparations, to work exclusively either with crude preparations, if this is feasible, or with salt free proteins. The latter method seems to be much safer, although it is, undoubtedly, more time consuming.

The purification of an enzyme preparation, as shown by the example of E 31 and E 311, may be satisfactorily followed by disc electrophoresis (Table 3).

Summarizing the results obtained it can be said that disc electrophoresis according to DAVIS (1964) is a suitable method to distinguish commercial milk clotting enzyme preparations of both animal and microbial origin or to follow the purification of a crude microbial preparation.

No group distinction between animal and microbial preparations is possible on the basis of the electrophoretograms: the representatives of either group have a well defined individual electrophoretic protein pattern including two fast moving bands which seem to characterize all the preparations of rennin activity tested.

Excessive salt content interferes with the electrophoretic separation and has to be removed previously by gel filtration.

The TRIS buffer, pH 8.3, used in this method inactivates both the milk clotting and proteolytic activities of most of the preparations. A more suitable buffer system allowing the estimation of the enzyme activities of the protein bands, could give additional information.

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## APPLICATION OF MEMBRANE SEPARATION PROCESSES TO THE CONCENTRATION OF DAIRY BY-PRODUCTS\*

M. KHELL-WICKLEIN

(Received September 22, 1972)

The importance of membrane separation techniques for the concentration of dairy by-products is pointed out and the results achieved with this method are summed up and evaluated.

The experiments carried out in the Central Food Research Institute, Budapest, with the aim to concentrate cheese whey are described. Cellulose acetate membranes have been developed for the separation and concentration of protein and lactose, resp., in whey and buttermilk. The characteristic features of these membranes are described, including their water permeability as a function of pressure.

An experimental equipment has been constructed which now operates in the Institute. The main chemical components of the produced concentrates and permeates are given.

The possibilities of membrane separation methods in the food industry, with particular respect to Hungarian conditions, are discussed.

Owing to the fact that no concentrate of sufficiently low water activity can be produced, in an economical way, by the membrane separation technique, such a process is bound to be combined with other methods of food preservation to produce microbiologically stable products.

In recent years, besides incessant improvement of traditional concentration methods, more and more attention has been paid to attempts aiming at the industrial introduction of concentration processes based on membrane separation.

The food industry is particularly interested in this new technology which is still in the development stage. Since it has been recognized that the method offers a possibility not only of reducing concentration costs, but also of producing concentrates of full biological value, several university and research laboratories are currently engaged in the investigation of the food industrial application of this technology, and several large engineering firms are working on the development of equipment best suited to food industrial requirements.

Of the membrane separation techniques mainly reverse osmosis and ultrafiltration promise to bring considerable progress into food industrial practice. The first may turn out to be a new economic method for the concentration of fruit and vegetable juices, while the second may greatly improve the manufacture of protein containing concentrates. In this capacity it could

\* Presented at the IUFOST Symposium on Combination Treatments in Food Preservation, Budapest, 18–22 September 1972.



play an important role in the combined preservation of foodstuffs by improving the economy of such modern, but costly procedures, as *e.g.* lyophilization which, when applied alone, is not profitable for the processing of liquid foods with low dry matter content.

In our opinion, within the food industry the dairy industry is perhaps the most interested in the utilization of membrane separation technologies. The up-to-date processing of those by-products of the dairy industry which contain valuable ingredients, *e.g.* of whey and buttermilk, without loss, is a task awaiting solution everywhere in the world. Despite the fact that in course of butter and cheese production a substantial part of the nutritive materials of milk pass into the whey and the buttermilk, so that their use in animal feed and, even more so, their conversion to human nourishment would result in a considerable rise in protein supplies, these materials are still treated in more than one place as waste. Because of their high biological index (50 000 to 60 000) their purification is a serious problem from the aspect of both the dairy industry and environmental protection (MARSHALL *et al.*, 1968; PORTER & MICHAELS, 1970). A survey of the American Dairy Science Association illustrates quite clearly the volume of assumedly lost nutrients: of the whey containing about 600 000 tons of dry matter not more than about 50% is industrially processed, while KEAY (1971) reports on a not more than about 40% processing of the whey into whey powder by the Canadian cheese industry.

Several factors hinder the direct utilization, and mainly the industrial processing, of dairy by-products.

From the aspect of utilization the low dry matter content of these liquids is particularly unfavourable (Table 1).

However, considering the main valuable components and the large volumes formed (Table 1), this relatively low dry matter content represents significant quantities of nutrients the biological value of which is further raised by their vitamin A, B<sub>1</sub>, B<sub>2</sub>, C and D, and pro-vitamin A and D contents.

Their direct utilization in animal feed cannot compete with that of other feeds because of the cumbersome and costly transport due to their high water content and can only be realized when fed in the vicinity of the dairy.

Table 1

*The main chemical components of whey and buttermilk*  
(SPREER, 1968; ZAJKOVSKIJ, 1953)

Components	Whey	Buttermilk
Water	93 — 94	90.5
Protein	0.8 — 1.0	3.2
Lactose	4.5 — 5.0	4.9
Ash	0.5 — 0.7	0.7



At present processing usually involves the one-step concentration of the whey and buttermilk and production of powder from the concentrate. When working with traditional concentration methods the production of concentrates and powders is not profitable because of the large quantities of water which have to be removed. In addition, the quality of proteins changes, denaturation takes place as a result of heat treatment. Only large plants, having always sufficient quantities of raw material available, can afford concentration by evaporation. In case of smaller plants transport costs contribute to the costs of evaporation, since, if the capacity of the plant is unable to ensure continuous operation of the concentrating equipment, this latter will be compelled to make up for lacking raw material by purchasing it from smaller dairies scattered over different and even far removed localities.

Utilization and processing is further encumbered by the fact that the storability of fresh whey and buttermilk is very limited. When stored at room temperature they will considerably deteriorate in the first 24 hours, so *e.g.* their lactose content may drop by as much as 20%, while pathogenic germs will find the conditions necessary for their growth. The use of preserving agents is not permitted in Hungary.

It follows from the aforesaid that the problem of the utilization of dairy by-products as human food or animal feed can be solved satisfactorily only by on the spot processing. Only in this way can deterioration of quality and loss during storage and transport be avoided and surplus costs of the anyhow problematic transport be eliminated.

Membrane separation processes appear to offer a highly advantageous solution for the decentralized concentration of whey and buttermilk. These processes which require electric energy only for the circulation of the liquor, can be realized under economic conditions in any small plant. Concentration equipment based on membrane separation can be adapted to the production volumes of plants of various capacities, its output can be flexibly varied and increased or reduced as required since it is built on the module principle. Concentration can be performed at any randomly chosen low temperature, so that the denaturation of proteins can be avoided, no unpleasant "cooked" taste will develop and the vitamins will be preserved without deterioration. Extensive reduction of the liquid volumes to be processed helps to observe during storage and transport the hygienic requirements of the manufacture of products for human consumption and results in a significant drop of transport costs. Finally, by reducing the BOD value of the removed liquid to a fraction of the original value and by removing the traces of fat from the feed, it greatly simplifies purification conditions.

Being aware of these advantages, today the dairy industry of the world turns with increasing attention to reverse osmosis and ultrafiltration, primarily of whey which represents the larger production volume. By means of reverse



osmosis it is possible to solve the joint concentration of all components of the dry matter in whey. Ultrafiltration enables the separation of whey protein from lactose and from the other components which is then followed by the separate concentration of the various fractions by means of reverse osmosis (McDONOUGH, 1968; MARSHALL *et al.*, 1968; SAMMON, 1969; GOLDSMITH *et al.*, 1970; McDONOUGH & MATTINGLY, 1970; BESIK *et al.*, 1971; PORTER & MICHAELS, 1971; HORTON *et al.*, 1970; McKENNA, 1970; FREEDMAN & SHABAN, 1971; OLSEN, 1971; WERNER & ARNOLD, 1971; WINGE, 1971).

In accordance with the objective to be achieved research has two main directions. On one hand, it endeavours to develop ultrafilter membranes which will retain proteins but permit the permeation of smaller molecules, while the other goal is the preparation of reverse osmosis membranes for the concentration of the latter substances. As witnessed by recently published, mainly American, reports and pamphlets, there are already membranes available which possess very good permeability and adequate selectivity to ensure the operation of large-scale plants. The dramatic developments in the plastic industry promise membranes with even better, improved properties.

With respect to the development of equipment types, those having permeators with tubular membranes are currently being preferred to plate membranes and to hollow fibre or spiral wound solutions. But for the type of membrane and applied pressure there is no essential difference between the one-step and two-step whey processing equipment. Equipments for two-step concentration are made up of two independent cycles which are capable of separate operations. Their capacities vary within wide limits. Thus *e.g.* the American firm ABCOR manufactures equipment for the concentration of 5 000 kg of whey, but is currently building a plant of 110 000 kg capacity for Crowley's Milk Company in New York (HORTON *et al.*, 1970).

According to literature data whey is concentrated to a dry matter content of 15 to 25% (WERNER & ARNOLD, 1971). WERNER and co-worker claim that the production of a 15% concentrate is more economic than that of a 30% concentrate. It is, however, possible to achieve a substantially higher dry matter content, thus, for instance, in the experiments performed by *Aerojet General* at 100 gauge pressure a concentrate with 56% dry matter content was produced, while DUNKLEY (1969) obtained a whey which was one tenth of its original volume.

In our institute we have been engaged since 1968 in the study of the applicability of membrane separation technologies in the food industry. As the first stage of our research we aimed at the home preparation of membranes for the separation and concentration of various substances and at the development of various equipment types. The concentration of dairy by-products has figured since two years on our programme.



## 1. Materials and methods

### 1.1. Materials

*1.1.1. Membranes.* Commercially available membranes and membranes prepared in our laboratory from cellulose acetate were used in the laboratory experiments. The latter were compared to commercially obtained membranes, that is to an Eastman Kodak HF-35 and a Sartorius SM 11736 membrane.

For the preparation of our membranes we used the method described in the literature (LOEB & SOURIRAJAN, 1964). By variation of the preparation parameters (composition of the casting mass, drawing layer thickness, evaporation time and temperature, time and temperature of heat treatment) membranes with different water permeabilities and selectivities were obtained, and of these some were chosen for trials with dairy by-products. These membranes bear the symbols: RÉKI-71/1, 71/2, 72/11 and 72/15.

### 1.1.2. Concentrated liquids

*1.1.2.1. — Whey.* Whey was prepared by using in a concentration of 60 mg per 1 litre of milk calf rennet customary in the dairy industry, having an activity of 9 000 SU (Soxhlet units) per gram. The whey obtained in this manner had a dry matter content of 6.5%, and a lactose content of 3.95%.

*1.1.2.2. — Buttermilk.* Buttermilk was the by-product of sour butter production; its analysis showed 6.0% of dry matter content and 2.90% of lactose content.

*1.1.2.3. — Lactose solution.* For the concentration of lactose a buttermilk permeate free of protein and obtained by ultrafiltration was used. The solution had a dry matter content of 2.5% and a lactose content of 2.2%.

*1.1.3. Concentration equipment.* For laboratory concentration experiments a stainless steel apparatus capable of treating 4 litre of the liquid and provided with an agitator was used. The useful membrane surface of the apparatus was 90 cm<sup>2</sup>. A nitrogen cylinder provided the pressure necessary for concentration.

### 1.2. Methods

*1.2.1. Determination of water permeability.* For the measurement of water permeability we built in our institute an apparatus which was suitable for the study of membranes of 7.07 cm<sup>2</sup> surface. Membrane performance was expressed in units of l/m<sup>2</sup> · day.

*1.2.2. Determination of selectivity.* Membrane selectivity was calculated from the lactose content of the input and of the permeate with the help of the



following formula:

$$V\% = \frac{C_1 - C_2}{C_1} \cdot 100,$$

where  $V$  = retention power,

$C_1$  = concentration of the input,

$C_2$  = concentration of the permeate.

1.2.3. *Determination of dry matter content.* The dry matter content of the liquids was determined gravimetrically.

1.2.4. *Determination of lactose content.* The reducing carbohydrate content of the liquids was determined by the Luff—Schoorl method.

## 2. Results

Fig. 1 illustrates the water permeability vs. pressure of one of our cellulose acetate membranes, compared to the same property of an Eastman Kodak and of a Sartorius dialyzing membrane.

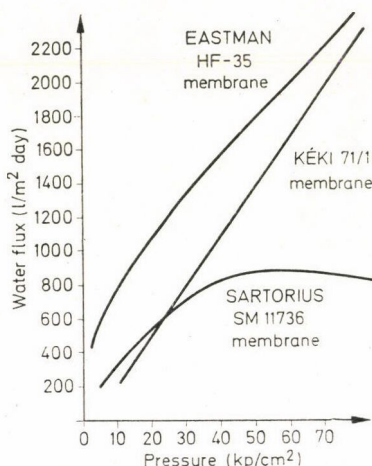


Fig. 1. Effect of operating pressure on pure water permeation using cellulose acetate membranes

In Table 2 we have summed up the water permeabilities of the membranes used in the experiments at 90 kpond/cm² gauge pressure and their selectivities for lactose.

The results of concentration experiments with whey and buttermilk, respectively, are given in Fig. 2.

Table 2

*Walter permeability and lactose retention of cellulose acetate membranes used in experiments for concentration*

Membrane	Pressure kp/cm <sup>2</sup>	Water permeability l/m <sup>2</sup> · day	Lactose retention %
KÉKI 71/1	90	2 400	96
KÉKI 71/2	90	1 280	81
KÉKI 72/11	60	2 470	16
KÉKI 72/15	60	4 500	24

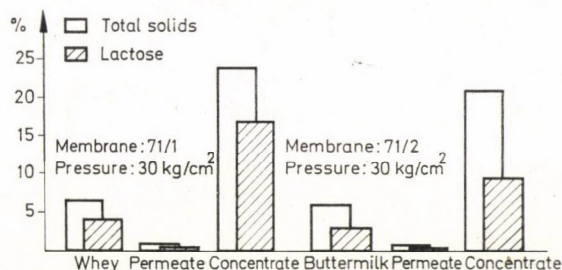


Fig. 2. Concentration of whey and buttermilk

Fig. 3 shows the results of two experiments in which separation of protein and lactose in whey and buttermilk, respectively, was attempted.

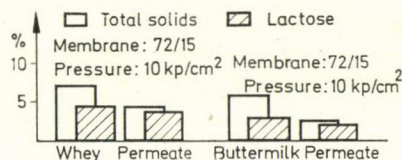


Fig. 3. Separation of lactose from whey and buttermilk by ultrafiltration

The permeate obtained by the experiment illustrated in Fig. 3 was free of protein, but contained 2.20% of lactose. This liquid was then further concentrated by means of a more lactose selective membrane in order to obtain this latter product. The results are shown in Fig. 4.

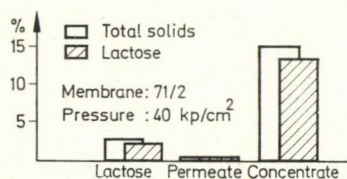


Fig. 4. Concentration of lactose (ultrafiltration permeate as feed)



### 3. Conclusions

From the results of experiments aiming at the concentration of dairy by-products and at the separation of their protein and lactose contents we have drawn the following conclusions:

In the investigated pressure range the water permeability of the Eastman Kodak membrane, as well as of the cellulose acetate membrane prepared in our institute increased continuously with increasing pressure, while the Sartorius membrane gave a maximum curve, indicating that under the applied experimental conditions there was practically no change in the structure of the first two membranes. The structure of the Sartorius membrane, on the other hand, deteriorated beyond a certain value of pressure and consequently its performance dropped too, with further increase of the pressure (Fig. 1).

Concentration experiments with whey and buttermilk resulted in concentrates with 20 to 24% of dry matter content. The effluent permeate was always water clear, contained no protein and its dry matter content was less than 0.8%. The whey concentrate of the experiment illustrated in Fig. 2 contained 24.0% of dry matter, 16.9% of lactose, while the dry matter and lactose content of the buttermilk preparation was 21.1% and 9.61%, resp.

By using membranes permeable to lactose, but impermeable to protein for the separation of protein and lactose it was possible to obtain the bulk of lactose in the permeate (Fig. 3). This permeate which was already free of protein was then concentrated by means of a lactose selective membrane and a concentrate with 14.9% of lactose was obtained (Fig. 4).

We observed the formation of a precipitate when working with concentrates having a dry matter content higher than 15.0%; this was particularly conspicuous in the case of buttermilk.

The experiments proved that vigorous agitation of the concentrated liquid is indispensable. In case of unsatisfactory agitation, beyond concentration polarization, a constantly thickening solid layer which adhered to the membrane also hindered the free movement of water through the membrane.

On the basis of literature data and of our own laboratory experiments we designed a pilot plant for the concentration of dairy by-products (Fig. 5) which currently operates with a tubular membrane of 0.2 m<sup>2</sup> surface. Its capacity depends on the properties of the membrane, on the quality, concentration and viscosity of the concentrated liquid, on the pressure and on several other, non-negligible factors. Work to increase the membrane surface is in progress and according to our calculations with a 1 m<sup>2</sup> membrane surface the equipment will produce daily about 180 to 200 litre of whey concentrate in which the dry matter content will be raised from 6% to 20%.

We have ascertained that in Hungary about 200 000 metric tons of whey are produced yearly, part of which is utilized as liquid feed. Whey powder is



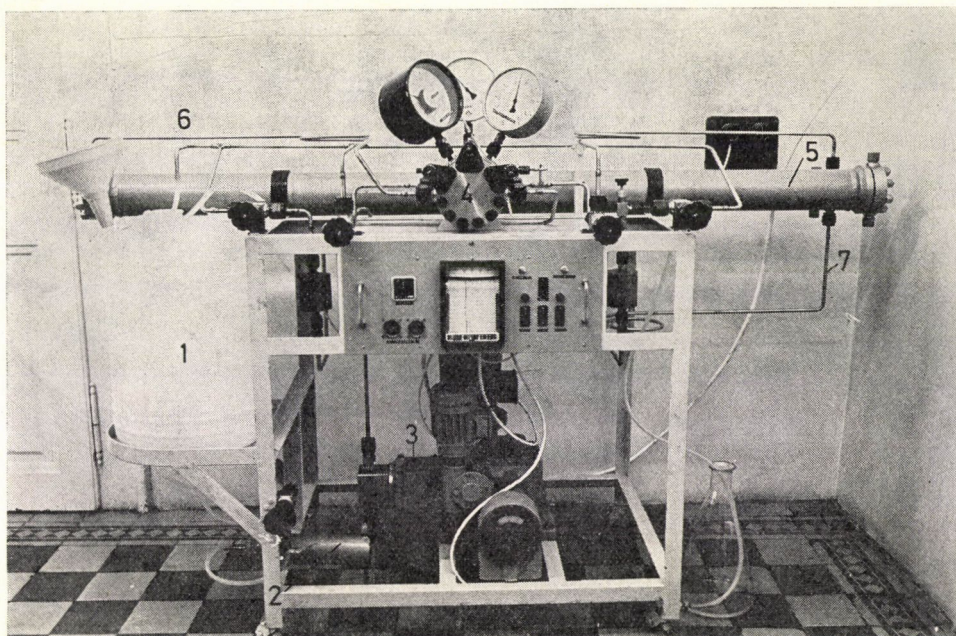


Fig. 5. Reverse osmosis equipment. 1. feed tank; 2. filter; 3. high pressure pump; 4. pressure regulator; 5. reverse osmosis unit; 6. product; 7. permeate

at present not manufactured in the country, though the increasing demand for whey powder will necessitate the establishment of suitable equipment. Our results, just as in the literature, seem to indicate that up-to-date and economic production of whey powder can be realized by the joint application of concentration by means of membrane separation, followed by some modern method of powder manufacture (lyophilization, spray drying, etc.). In our opinion an equipment of 10 to 20 thousand litre daily capacity would solve the preliminary concentration of dairy by-products to a 20 to 25% dry matter content on the spot. The concentrate produced in this manner can then be transported in refrigerated state to the central powder producing plants for further processing. Whey powder produced under hygienic conditions can be utilized not only as animal feed, but also by the food industry, mainly as an additive, and also by the industries for pharmaceutical and dietetic products as a basic ingredient with valuable protein content.

We believe that in the near future membrane separation processes will have attained an important role in combined preservation. These are efficient and economic methods for the concentration of liquids with low dry matter contents, while their application in the food industry may lead to the saving of nutrients which before were treated as wastes.



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## CONCENTRATION OF FRUIT JUICES BY REVERSE OSMOSIS USING POROUS CELLULOSE ACETATE MEMBRANES\*

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The physicochemical criteria for the reverse osmosis separation of inorganic ions, alcohols, aldehydes, ketones, esters, acids, sugars and nonpolar substances are discussed and illustrated with particular reference to the separation of fruit juice components using porous cellulose acetate membranes. Equations of reverse osmosis transport applicable to the processing of fruit juices are given, and their applicability for the determination of the osmotic pressures of fruit juice solutions are discussed and illustrated. Experimental data on the osmotic pressure of commercially available apple juice, pineapple juice, orange juice, grapefruit juice, grape juice, and tomato juice and their concentrations are given. The applicability of system analysis for the reverse osmosis concentration of fruit juices is discussed and illustrated. Experimental data on the processing capacities of some typical cellulose acetate membranes for the concentration of above juices are given.

The application of reverse osmosis for fruit juice concentration is of both scientific and technical interest. In the literature, there are several reports (MERSON & GINETTE, 1970, 1972; MERSON, 1969; MERSON *et al.*, 1969; MERSON & MORGAN, 1968; PORTER & MICHAELS, 1971) which discuss the operational aspects of reverse osmosis for food processing including fruit juice concentration. These reports are based on empirical concepts of semipermeability of membranes in reverse osmosis. The potential practical importance of reverse osmosis to the field of food technology in general, and fruit juice concentration in particular, calls for a more fundamental approach to the subject. From this point of view, the subject is currently wide open, and calls for extensive investigations. It is the object of this paper to initiate such investigations and call attention to some fundamental aspects of the subject.

The subject is primarily concerned with the basic physicochemical criteria for the reverse osmosis separation of fruit juice components, equations of reverse osmosis transport applicable to the concentration of fruit juices, the experimental technique for determining the osmotic pressure of fruit juices and their concentrates, and the engineering science of reverse osmosis process design for the particular application under discussion. This paper makes a contribution to each one of the above aspects of reverse osmosis with particular

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reference to its application for fruit juice concentration using the Loeb-Sourirajan type porous cellulose acetate membranes.

### 1. Materials and methods

Two types of reverse osmosis cells, flow-type and nonflow-type, were used in this work. Details of cell design and experimental procedures used are the same as those given in the literature (SOURIRAJAN, 1970a). The nonflow-type cell (effective film area = 9.6 cm<sup>2</sup>) was used to determine the osmotic pressure of fruit juices, and their concentration to different levels of product recovery. The flow-type cell (effective area = 7.6 cm<sup>2</sup>) was used for all other experiments. Laboratory made Batch 316-type porous cellulose acetate membranes (KUNST & SOURIRAJAN, 1970) and commercially available apple juice (Allens), pineapple juice (Dole), orange juice (Libbys), grapefruit juice (Libbys), grape juice (Welchs), and tomato juice (Heinz) were used in this work. The pineapple juice and orange juice were initially filtered through a cloth filter before use in reverse osmosis experiments. The system sodium chloride-water was used to obtain data on specifications of membranes and feed flow conditions (SOURIRAJAN, 1970b). The specifications of all the membranes referred to in this work are given in Table 1 in terms of data on pure water permeability constant,  $A$  (in gram moles water/cm<sup>2</sup> sec atm) and solute transport parameter,  $(D_{AM}/K\delta)$  for sodium chloride (in cm/sec) at the indicated operating pressures. Some data on membrane performance for the system sodium chloride-water are also given in Table 1 for the specified feed solutions and mass transfer coefficients,  $k$  (in cm/sec), on the high pressure side of the membrane. All experiments were carried out at the laboratory temperature (23–25 °C). The reported product rates are those corrected to 25 °C using the relative viscosity and density for pure water. In all experiments, the terms "product" and "product rate" refer to membrane permeated solutions. For single solute systems, the fraction solute separation ( $f$ ) was defined as

$$f = \frac{\text{solute ppm in feed} - \text{solute ppm in product}}{\text{solute ppm in feed}} \quad (1)$$

and for fruit juices, the fraction solute separation was defined in terms of carbon content of solutions as

$$f = \frac{\text{carbon ppm in feed} - \text{carbon ppm in product}}{\text{carbon ppm in feed}} \quad (2)$$

The Beckman total carbon analyzer, model 915, was used to measure the carbon content of organic solutes in the feed, product and concentrated



solutions. The concentration of sodium chloride in aqueous solutions was determined using a conductivity bridge.

## 2. Results

### 2.1. *Physicochemical criteria for reverse osmosis separation*

2.1.1. *Mechanism of reverse osmosis.* The origin of the reverse osmosis process as it is practiced to-day, is the result of the recognition of a fundamental concept, namely that an appropriate chemical nature of the membrane surface in contact with the solution as well as the existence of pores of appropriate size on the area of the membrane at the interface together constitute the indispensable twin requirement for the industrial success of this separation process. This concept, called the preferential sorption-capillary flow mechanism, is discussed in detail in the literature (SOURIRAJAN, 1970a, b, 1972). According to this mechanism, reverse osmosis separation is governed, in part, by a surface (interfacial) phenomenon; the surface layer of a successful reverse osmosis membrane is microporous at all levels of solute separation; and, if only the membrane material (*i.e.*, the material of the membrane surface) has a preferential sorption for water (or preferential repulsion for solute) under the conditions of the experiment, practically any degree of solute separation can be obtained by simply changing the pore size on the membrane surface. On the other hand, for a given pore size on the membrane surface, under otherwise identical experimental conditions, a higher solute separation in reverse osmosis means that the membrane material has a greater preferential sorption for water with respect to the above solute relative to others under comparison.

The above mechanism gives rise to the concept of the existence of a critical pore diameter on the membrane surface for maximum solute separation and fluid permeability. This diameter is obviously twice the thickness of the preferentially sorbed interfacial pure water layer. Since the magnitude of the latter could be different for different membrane-solution-operating conditions, even when the membrane material has a preferential sorption for water, the level of solute separation will be different for different solutes for a given pore diameter on the membrane surface. The performance of the membrane in reverse osmosis (*i.e.*, solute separation and product rate) depends on the number, size and size-distribution of pores on the membrane surface, in addition to the chemical nature of membrane material and feed solution, and the other operating conditions of the experiment.

From the above considerations, it is also clear that when the membrane material has no preferential sorption for water with respect to any particular solute, there can be no solute separation in reverse osmosis whatever be the



Table 1  
Specification of membranes

Film no.	1	3	28	29	J1	J2	J3
Film shrinkage temperature, °C	81	75	81	73	80	80	80
<i>Specification at operating pressure</i>							
Pure water permeability constant, $A \left( \frac{\text{g-mole}}{\text{cm}^2 \cdot \text{sec} \cdot \text{atm}} \right) \times 10^6$	2.48	5.35	3.11	5.87	2.00	2.28	1.78
Solute transport parameter $(D_{AM}/K\delta)_{\text{NaCl}} (\text{cm/sec}) \times 10^5$	3.22	16.89	4.17	24.85	4.21	4.59	2.68
<i>Performance data</i>							
Feed concentration, ppm NaCl	1 500	1 500	1 500	1 500	5 000	5 000	5 000
Mass transfer coefficient, $k$ (cm/sec) $\times 10^4$	57	57	43	43	20	20	20
Operating pressure, psig	250	250	250	250	1 000	1 000	1 000
Solute separation, %	94.7	90.4	93.8	83.5	93.5	92.0	95.0
Product rate, g/hr	19.1*	41.2*	23.8*	45.5*	69.2**	74.5**	60.9**

\* Flow-type cell, film area 7.6 cm<sup>2</sup>

\*\* Nonflow-type cell, film area 9.6 cm<sup>2</sup>

porous structure of the membrane surface. Further, if, under a particular set of conditions, the membrane material has a preferential sorption for solute instead of water, then the solute may pass through the membrane pores preferentially resulting in solute enrichment (*i.e.*, negative solute separation) in the product solution.

Therefore, in dealing with an aqueous feed solution containing different solutes (either in single species or in admixture) in conjunction with a particular membrane in the reverse osmosis process, the question is not whether the membrane is "semipermeable" or not; but the question is two-fold, namely, whether the membrane material has a preferential sorption for water with respect to each one of the solutes under consideration, and, with the existing pore structure on the membrane surface, what will be the product rate and solute separation with respect to each solute under consideration at the preset operating conditions of the experiment. The answer to the latter set of questions, with respect to solute separation, depends on the physicochemical criteria for preferential sorption of water with respect to different solutes and the effect of operating conditions on solute separation. These aspects are discussed below with particular reference to Loeb-Sourirajan type porous cellulose acetate

and some performance data

J4	J5	J6	J7	J8	J9	J10	J11	J12	J13
80	80	80	80	80	80	80	80	80	80
2.54	2.92	3.04	2.40	2.48	2.31	2.28	1.89	2.05	2.08
14.76	14.15	13.27	1.44	5.33	5.36	4.17	3.69	4.45	2.35
5 000	5 000	5 000	5 000	5 000	5 000	5 000	5 000	5 000	3 500
20	20	20	20	20	20	20	20	20	50
1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000
84.1	85.7	88.5	98.0	91.1	93.2	94.5	95.8	93.2	98.4
89.4**	102.0**	107.4**	82.2**	81.1**	81.0**	79.2**	69.6**	71.1**	65.2*

membranes and aqueous feed solutions containing single solutes which are commonly present in fruit juices.

*2.1.2. Major components in solution in fruit juices.* From the point of view of this work, the water soluble components in fruit juices may be considered to consist essentially of 10 to 20% of sugars, ~1% of acids, <1% of inorganics, and very small quantities of volatile flavour compounds, vitamins, fats and proteins (Table 2). Therefore it would be of interest to know the physicochemical criteria governing the reverse osmosis separation of all the above classes of compounds. Since sugars constitute the bulk of the dissolved material, highest possible solute separation with respect to sugars, and hence their practically complete retention in the concentrate, is a necessary criterion for the choice of the porous structure of the membrane for use in the reverse osmosis process. This criterion alone is insufficient for the practical success of reverse osmosis for fruit juice concentration; the process must also be capable of separating and concentrating a major part of the other components present in the fruit juices. This latter requirement is particularly important with respect to the separation and concentration of the volatile flavour compounds present in fruit juices. These latter compounds consist essentially of a mixture



of a large number of alcohols, aldehydes, ketones, esters and acids. For this reason, the physicochemical criteria for the reverse osmosis separation of such flavour compounds are of major interest in this work. Even though the physicochemical criteria for the reverse osmosis separation of all classes of solutes for different membrane materials and aqueous feed solutions have not yet been developed, a consistent set of criteria has already emerged with respect to the reverse osmosis separation of inorganics, alcohols, aldehydes, ketones, esters, acids and sugars in aqueous solutions, using the Loeb-Sourirajan type porous cellulose acetate membranes. These criteria are summarized and their applications illustrated in Table 2 with particular reference to the major components of fruit juice solutions.

*2.1.3. Separation of inorganic ions.* This is probably not a critical factor in determining the applicability of the reverse osmosis process for fruit juice

Table 2  
*Major components in solution in fruit juice\**

Component	Apple juice	Pineapple juice	Orange juice	Grapefruit juice	Grape juice	Tomato juice
<i>Sugars, wt. %</i>						
D-Glucose	1.3-2.0	2.1-2.4	2.6-5.8	3.4-5.0	11.5-19.3	~4.3
D-Fructose	4.4-8.2	2.1-2.4			—	
Sucrose	1.7-4.2	8.4-9.5	3.1-5.1	1.3-3.0	0.2-2.3	
<i>Acids, wt. %</i>						
L-Lactic acid	present				present	0.2-0.6*
D-Malic acid	0.3-1.0	0-0.2			present	
Citric acid	0-0.03	0.7-0.9	0.4-1.5*	0.9-1.4	0.7-1.7	
Tartaric acid	present		present			
<i>Volatile flavour compounds, ppm</i>						
Alcohols	46	present				(not reported)
Aldehydes	3	present				
Ketones		present	present	(not reported)	present	
Esters	1	22-414				
Acids		18-118				
Hydrocarbons		present				
<i>Fats, wt. %</i>			0.2-0.5	~0.1		~ 0.2
<i>Proteins, wt. %</i>		0.4-0.5	0.6-0.8	0.3-0.6	0.2-0.9	~ 1.0
<i>Vitamins, ppm</i>		110-116	300-800			~160
<i>Inorganics, wt. %</i>		0.2-0.5	0.5-0.9	0.2-0.4	0.3-0.4	~ 1.0
Water, wt. %	80 to 95					

\* Data from TRESSLER & JOSLYN (1971)

concentration. However, the physicochemical criteria governing the separation of inorganic ions are of critical importance in view of the applicability of the principles involved for the separation of all ionic species including partly dissociated fruit juice acids.

Following the basic mechanism of reverse osmosis referred to above, GLUECKAUF (1965) and BEAN (1969) showed analytically that ions are electrostatically repelled in the vicinity of membrane materials of low dielectric constant under conditions of reverse osmosis operation. *Glueckauf's* analysis predicts that solute separation

decreases with increasing pore radius on membrane surface,  
decreases with increasing ionic radius,  
decreases with increasing solute concentration in feed,  
increases with decreasing dielectric constant of membrane material, and  
increases with increasing ionic charge.

*Bean's* analysis confirms and extends the results of *Glueckauf's* analysis, and includes the effect of operating pressure on solute separation. *Bean* calculated that for a membrane material of dielectric constant 3 and a dilute solution of 1—1 electrolyte, solute separations of 90, 99 and 99.9% could be obtained from 27 Å, 13.5 Å and 9 Å pores, respectively. His results also show that for 99% solute separation, the pore sizes could be 27 Å for 2—1 electrolyte and 40 Å for 2—2 electrolyte with a membrane material of dielectric constant 3. On the other hand, with a 27 Å pore in a membrane material of dielectric constant 10, the obtainable solute separations are about 60, 83 and 97% for 1—1, 2—1, and 2—2 electrolytes, respectively, under otherwise identical operating conditions. Since cellulose acetate has a dielectric constant of 4 to 5.5, and since both monovalent and divalent ions have been identified in fruit juices (TRESSLER & JOSLYN, 1971), the results of *Beans's* calculations are of practical interest. The analyses of *Glueckauf* and *Bean* show that dielectric constant of membrane material, ionic size and ionic charge are important physicochemical criteria governing the separation of inorganic ions in reverse osmosis; *Bean's* results further show how the above criteria determine the membrane pore size necessary to obtain a desired level of solute separation in the reverse osmosis process.

The analysis of GLUECKAUF (1965) and BEAN (1969) are for neutral membrane surfaces; if the latter have fixed charges, additional effects should be expected. When a charged microporous body is in equilibrium with an external electrolyte solution, the co-ions (*i.e.*, ions, the charge of which has the same sign as that of the fixed charge) are repelled from, and the counter ions attracted to the membrane surface setting up an electrical double layer and hence a concentration gradient on the membrane surface. The methods of calculating the



consequent changes in ionic concentrations in the microporous medium are discussed extensively by DRESNER (1963a, b, 1965; DRESNER & KRAUS, 1963).

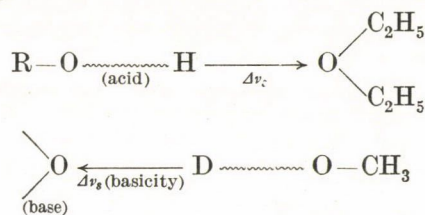
Reverse osmosis membranes may have neutral or charged surfaces. The surface of a cellophane membrane (cellulose material) carries a negative charge according to HEYMANN and RABINOV (1941) and VAN OSS (1963, 1970). Even though cellulose acetate material is not generally considered to have any fixed charges, results of reverse osmosis experiments indicate that the surface of a cellulose acetate membrane behave as if it carried some negative charge.

Cellulose acetate membranes with surface pore structures capable of giving over 99% solute separation for sodium chloride are currently available (GILLAM & PODALL, 1970). If the objective is simply to obtain more than 99% solute separation with respect to sugars, a pore size on the membrane surface corresponding to 99% level of solute separation for sodium chloride will not be necessary; a larger pore size will be adequate for the purpose. Consequently, neither the physicochemical criteria governing the separation of inorganic ions, nor the current level of membrane technology limits the choice of the porous structure of cellulose acetate membranes for fruit juice concentration with respect to inorganics and sugars.

*2.1.4. Separation of alcohols, aldehydes, ketones and esters.* The physicochemical criteria governing the reverse osmosis separation of these classes of solutes in aqueous solution using porous cellulose acetate membranes have recently been studied and reported (MATSUURA & SOURIRAJAN, 1971b, 1972a, b, 1973). The alcohols, aldehydes, ketones and esters are present in aqueous solution essentially as undissociated molecules. It has been shown that under such conditions, preferential sorption of water on the membrane surface, and hence solute separation in reverse osmosis, is governed by the polar effect of the molecule. The latter can be represented by the hydrogen bonding ability of the solute molecule as given either by the stretching of the OH or OD bond corresponding to the incipient ionization of the molecule, or by Taft number (TAFT, 1956) of the substituent group in the solute molecule with respect to each functional group.

Hydrogen bonding represents the tendency of proton transfer from an acid (proton donor) to a base (proton acceptor). The hydrogen bonding ability of alcohols (proton donors, acids) was determined quantitatively by measuring the shift ( $\Delta\nu_s$ ,  $\text{cm}^{-1}$ ) in the OH band maximum in IR spectra of the solute in  $\text{CCl}_4$  and ether solutions; the hydrogen bonding ability of aldehydes, ketones and esters (proton acceptors, bases) was determined quantitatively by the shift ( $\Delta\nu_s$  (basicity),  $\text{cm}^{-1}$ ) in the OD band maximum in the IR spectra of  $\text{CH}_3\text{OD}$  in benzene and the particular proton accepting solvent. The hydrogen bonding situation in the experimental measurements of  $\Delta\nu_s$  and  $\Delta\nu_s$  (basicity) may be

represented as follows:



Values of  $\Delta\nu_s$  give a relative measure of the proton donating power of the acid (alcohols) and those of  $\Delta\nu_s$  (basicity) give a similar measure of the proton accepting power of the base molecules (aldehydes, ketones and esters). Increase in  $\Delta\nu_s$  and that in  $\Delta\nu_s$  (basicity) both represent an increase in the hydrogen bonding ability with respect to the molecules concerned. Data on solute separations using porous cellulose acetate membranes with respect to a large number of alcohols, aldehydes, ketones and esters were correlated with their corresponding data on  $\Delta\nu_s$  or  $\Delta\nu_s$  (basicity), the results showed that solute separation increased with decrease in  $\Delta\nu_s$  or increase in  $\Delta\nu_s$  (basicity) (MATSUURA & SOURIRAJAN, 1971b, 1972a, b, 1973). These results are extremely significant. They show (i)  $\Delta\nu_s$  and  $\Delta\nu_s$  (basicity) are physico-chemical parameters governing the reverse osmosis separation of alcohols, aldehydes, ketones and esters; (ii) with reference to the above solutes, an increase in the hydrogen bonding ability results in a decrease in solute separation if the solute molecule is an acid (proton donor) or an increase in solute separation if the solute molecule is a base (proton acceptor); (iii) with respect to solute separation in reverse osmosis, an increase in  $\Delta\nu_s$  is equivalent to a decrease in  $\Delta\nu_s$  (basicity); (iv) the cellulose acetate membrane material has a net basic character, *i.e.*, the membrane surface attracts proton donor solutes and repels proton acceptor solutes, and (v) in the system alcohol-water, it is necessary that  $\Delta\nu_s$  for alcohol be less than that for water for the latter to be preferentially sorbed at the membrane-solution interface.

The significance of Taft number ( $\sigma^*$ ) with reference to reverse osmosis separation has been discussed (MATSUURA & SOURIRAJAN, 1971b). The Taft number gives a measure of the polar effect of the substituent group in a molecule. Since polar effects are additive for polysubstituted derivatives, the total polar effect of the substituent groups is given by the sum of their respective  $\sigma^*$  values; this sum is represented by  $\Sigma\sigma^*$ . Recently an additive technique has also been developed to assign  $\Sigma\sigma^*$  values for polyhydric alcohols (MATSUURA & SOURIRAJAN, 1973). The Taft number ( $\sigma^*$  or  $\Sigma\sigma^*$ ) is a measure of the electron withdrawing power of the substituent group or groups in a polar molecule. Hence a decrease in the value of  $\sigma^*$  or  $\Sigma\sigma^*$  is equivalent to a decrease in acidity or increase in basicity of the molecule. Consequently, with respect to both proton donor (acid) and proton acceptor (base) solutes, an increase in



solute separation can be expected with decrease in  $\sigma^*$  or  $\Sigma\sigma^*$ . Such correlations have been consistently established with respect to the separation of alcohols, aldehydes, ketones and esters (MATSUURA & SOURIRAJAN, 1971b, 1972a).

An analysis of the experimental reverse osmosis data for the separation of alcohols, aldehydes, ketones and esters present in very low concentrations in aqueous feed solutions, shows that the solute transport parameter ( $D_{AM}/K\delta$ ) (calculated from *Kimura—Sourirajan* equations) (SOURIRAJAN, 1970b), and the Taft number are related by the expression:

$$(D_{AM}/K\delta) = C \exp(\varrho^* \Sigma\sigma^*), \quad (3)$$

where  $\varrho^*$  is a polar functional constant applicable to reverse osmosis transport (obtained from the slope of the  $\Sigma\sigma^*$  versus  $\log(D_{AM}/K\delta)$  correlation), and  $C$  is a proportionality constant depending on the porous structure of the membrane. The value of  $\varrho^*$  is independent of the porous structure of the membrane, but depends on the nature of the functional group in the molecule; and, even for the same functional group, the values of  $\varrho^*$  are different for different ranges of  $\Sigma\sigma^*$ . Further, on the basis of the Taft equation (TAFT, 1956),  $\varrho^*$  should also be expected to depend on the reverse osmosis operating conditions such as pressure, temperature, nature of solvent and chemical nature of membrane. When the applicable value of  $\varrho^*$  is known, equation 3, along with the general *Kimura—Sourirajan* equations for reverse osmosis transport (SOURIRAJAN, 1970b), offers a means of predicting solute separation for all alcohols, aldehydes, ketones and esters from only a single set of experimental data for any one solute (taken as reference solute) in each functional group. This prediction procedure has been illustrated in detail in an earlier paper (MATSUURA & SOURIRAJAN, 1973). With particular reference to aqueous feed solutions containing very low concentrations of alcohols, aldehydes, ketones and esters, the above prediction procedure involves the following steps:

Determine  $\Sigma\sigma^*$  and ( $D_{AM}/K\delta$ ) for the reference solute.

Generate the  $\log(D_{AM}/K\delta)$  versus  $\Sigma\sigma^*$  correlation using the applicable value of  $\varrho^*$  for the particular experimental conditions and the desired range of  $\Sigma\sigma^*$  values.

From the above correlation, find the ( $D_{AM}/K\delta$ ) value for the desired solute whose  $\Sigma\sigma^*$  value is known.

Determine the mass transfer coefficient  $k$  on the high pressure side of the membrane applicable for the solute at the chosen reverse osmosis operating conditions.

Calculate solute separation  $f$  from the following relation:

$$f = 1 \left/ \left( \frac{D_{AM}}{K\delta} \right) \frac{\exp \left\{ \frac{[PR]}{3600 S k d} \right\}}{\left( \frac{[PR]}{3600 S d} \right)} \right. + 1 \quad (4)$$

where  $[PR]$  = product rate in grams per hour for the given membrane area of  $S$  cm<sup>2</sup>, and  $d$  = density of solution in grams per cm<sup>3</sup>, and  $(D_{AM}/K\delta)$  and  $k$  are expressed in units of cm per sec.

Equations 3 and 4 are particularly useful for predicting the relative separation of flavour compounds normally present in fruit juices, in reverse osmosis operation. This is illustrated in Fig. 1 with respect to the performance of two samples of membranes (films 1 and 3) of different surface porosities at an operating pressure of 250 psig.

Table 3 gives a partial list of fruit juice flavour compounds together with their Taft numbers. The compounds listed include 15 alcohols, 7 aldehydes, 3 ketones and 45 esters all of which have been identified in natural fruit juices (TRESSLER & JOSLYN, 1971). The ranges of  $\Sigma\sigma^*$  values for the above compounds are 0 to  $-0.230$  for alcohols,  $0.49$  to  $-0.134$  for aldehydes,  $0$  to  $-0.200$  for ketones and  $0.50$  to  $-0.380$  for esters.

For the porous cellulose acetate membranes of the type used in this work, at an operating pressure of 250 psig, the values of  $\rho^*$  for alcohols, aldehydes, ketones and esters in different ranges of  $\Sigma\sigma^*$  values have been calculated from the experimental reverse osmosis data reported earlier (MATSUURA & SOURIRAJAN, 1971b, 1972a), and the results are given in Table 4. Assuming that the  $\rho^*$  value of 1.49 is valid for esters up to a  $\Sigma\sigma^*$  value of  $-0.38$ , Table 4 gives the applicable  $\rho^*$  values for all the alcohols, aldehydes, ketones and esters listed in Table 3.

Using the above values of  $\rho^*$  and the experimental reverse osmosis data for the reference solutes specified in Table 4, the solute separations obtainable with films 1 and 3 for all the alcohols, aldehydes, ketones and esters listed in Table 3 were calculated and the results are given in Fig. 1 for three arbitrarily chosen values of  $k$ , namely  $\infty$ ,  $50 \times 10^{-4}$ , and  $10 \times 10^{-4}$  cm/sec. In these calculations the solute concentrations were assumed to be very low so that product rates were unaffected by osmotic pressure considerations, and the values of  $[PR]$  used were 23 and 48 grams per hour, respectively, for films 1 and 3 for a membrane area ( $S$ ) of 7.6 cm<sup>2</sup>.

Several aspects of Fig. 1 are significant. For the membrane-solution-operating conditions under consideration, positive solute separation is obtainable with respect to all the solutes listed in Table 3. The extent of solute sep-



Table 3  
Taft numbers for some fruit juice components

Solute		Taft number $\sigma^*$ or $\Sigma\sigma^*$
Name	Formula	
<i>Alcohols</i>	R in R—OH	
2-Pentanol	2-C <sub>5</sub> H <sub>11</sub>	-0.230
s-Butyl alcohol	s-C <sub>4</sub> H <sub>9</sub>	-0.210
i-Propyl alcohol	i-C <sub>3</sub> H <sub>7</sub>	-0.190
n-Hexyl alcohol	n-C <sub>6</sub> H <sub>13</sub>	-0.134
n-Octyl alcohol	n-C <sub>8</sub> H <sub>17</sub>	-0.134
n-Nonyl alcohol	n-C <sub>9</sub> H <sub>19</sub>	-0.134
n-Decyl alcohol	n-C <sub>10</sub> H <sub>21</sub>	-0.134
n-Pentanol	n-C <sub>5</sub> H <sub>11</sub>	-0.133
n-Butyl alcohol	n-C <sub>4</sub> H <sub>9</sub>	-0.130
i-Butyl alcohol	i-C <sub>4</sub> H <sub>9</sub>	-0.125
n-Propyl alcohol	n-C <sub>3</sub> H <sub>7</sub>	-0.115
Ethyl alcohol	C <sub>2</sub> H <sub>5</sub>	-0.100
	CH <sub>3</sub>	
2-Methylbutan-1-ol	CH <sub>3</sub> CH <sub>2</sub> CHCH <sub>2</sub> —	-0.075
3-Methylbutan-1-ol	CH <sub>3</sub> CHCH <sub>2</sub> CH <sub>2</sub> —	-0.045
	CH <sub>3</sub>	
Methyl alcohol	CH <sub>3</sub>	0
(Water)	H	0.49
<i>Aldehydes</i>	R in R—C(=O)H	
1-Octanal	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>6</sub>	-0.134
1-Nonanal	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>7</sub>	-0.134
1-Decanal	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub>	-0.134
1-Undecanal	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>9</sub>	-0.134
1-Hexanal	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub>	-0.133
Acetaldehyde	CH <sub>3</sub>	0
Formaldehyde	H	0.490
<i>Ketones</i>	R <sub>1</sub> , R <sub>2</sub> in R <sub>1</sub> —C(=O)—R <sub>2</sub>	
3-Pentanone	C <sub>2</sub> H <sub>5</sub> , C <sub>2</sub> H <sub>5</sub>	-0.200
2-Pentanone	CH <sub>3</sub> , C <sub>3</sub> H <sub>7</sub>	-0.115
Acetone	CH <sub>3</sub> , CH <sub>3</sub>	0
<i>Esters</i>	R <sub>1</sub> , R <sub>2</sub> in R <sub>1</sub> —C(=O)—O—R <sub>2</sub>	
2-Propyl 2-methylpropionate	i-C <sub>3</sub> H <sub>7</sub> , i-C <sub>3</sub> H <sub>7</sub>	-0.380
2-Propyl caproate	n-C <sub>5</sub> H <sub>11</sub> , i-C <sub>3</sub> H <sub>7</sub>	-0.323
Ethyl 2-methylbutyrate	s-C <sub>4</sub> H <sub>9</sub> , C <sub>2</sub> H <sub>5</sub>	-0.310
2-Propyl butyrate	n-C <sub>3</sub> H <sub>7</sub> , i-C <sub>3</sub> H <sub>7</sub>	-0.305
2-Propyl propionate	C <sub>2</sub> H <sub>5</sub> , i-C <sub>3</sub> H <sub>7</sub>	-0.290
Ethyl 2-methylpropionate	i-C <sub>3</sub> H <sub>7</sub> , C <sub>2</sub> H <sub>5</sub>	-0.290
Pentyl hexanoate	n-C <sub>5</sub> H <sub>11</sub> , n-C <sub>5</sub> H <sub>11</sub>	-0.266
n-Butyl caproate	n-C <sub>5</sub> H <sub>11</sub> , n-C <sub>4</sub> H <sub>9</sub>	-0.263
n-Butyl butyrate	n-C <sub>3</sub> H <sub>7</sub> , n-C <sub>4</sub> H <sub>9</sub>	-0.245
Ethyl heptanoate	n-C <sub>6</sub> H <sub>13</sub> , C <sub>2</sub> H <sub>5</sub>	-0.234
Ethyl octanoate	n-C <sub>7</sub> H <sub>15</sub> , C <sub>2</sub> H <sub>5</sub>	-0.234

Table 3 (cont.)

Solute		Taft number $\sigma^*$ or $\Sigma\sigma^*$
Name	Formula	
Ethyl decanoate	$n\text{-C}_9\text{H}_{19}, \text{C}_9\text{H}_5$	-0.234
Ethyl caproate	$n\text{-C}_5\text{H}_9, \text{C}_2\text{H}_5$	-0.233
n-Butyl propionate	$\text{C}_2\text{H}_5, n\text{-C}_4\text{H}_9$	-0.230
Ethyl pentanoate	$n\text{-C}_4\text{H}_9, \text{C}_2\text{H}_5$	-0.230
Ethyl 3-methylbutyrate	$i\text{-C}_4\text{H}_9, \text{C}_2\text{H}_5$	-0.225
Ethyl butyrate	$n\text{-C}_3\text{H}_7, \text{C}_2\text{H}_5$	-0.215
Methyl 2-methylbutyrate	$s\text{-C}_4\text{H}_9, \text{CH}_3$	-0.210
Ethyl propionate	$\text{C}_2\text{H}_5, \text{C}_2\text{H}_5$	-0.200
2-Propyl acetate	$\text{CH}_3, i\text{-C}_3\text{H}_7$	-0.190
Methyl 2-methylpropionate	$i\text{-C}_3\text{H}_7, \text{CH}_3$	-0.190
Methyl heptanoate	$n\text{-C}_6\text{H}_{13}, \text{CH}_3$	-0.134
Methyl octanoate	$n\text{-C}_7\text{H}_{15}, \text{CH}_3$	-0.134
Methyl decanoate	$n\text{-C}_9\text{H}_{19}, \text{CH}_3$	-0.134
n-Hexyl acetate	$\text{CH}_3, n\text{-C}_6\text{H}_{13}$	-0.134
Methyl caproate	$n\text{-C}_5\text{H}_{11}, \text{CH}_3$	-0.133
n-Butyl acetate	$\text{CH}_3, n\text{-C}_4\text{H}_9$	-0.130
Methyl pentanoate	$n\text{-C}_4\text{H}_9, \text{CH}_3$	-0.130
2-Methyl-1-propyl acetate	$\text{CH}_3, i\text{-C}_4\text{H}_9$	-0.125
Methyl 3-methylbutyrate	$i\text{-C}_4\text{H}_9, \text{CH}_3$	-0.125
1-Propyl acetate	$\text{CH}_3, n\text{-C}_3\text{H}_7$	-0.115
Methyl butyrate	$n\text{-C}_3\text{H}_7, \text{CH}_3$	-0.115
Ethyl acetate	$\text{CH}_3, \text{C}_2\text{H}_5$	-0.100
Methyl propionate	$\text{C}_2\text{H}_5, \text{CH}_3$	-0.100
2-Methyl-1-butyl acetate	$\text{CH}_3, s\text{-C}_4\text{H}_9(\text{CH}_2)$	-0.075
3-Methyl-1-butyl acetate	$\text{CH}_3, i\text{-C}_4\text{H}_9(\text{CH}_2)$	-0.045
Methyl 4-methylpentanoate	$i\text{-C}_4\text{H}_9(\text{CH}_2), \text{CH}_3$	-0.045
Methyl acetate	$\text{CH}_3, \text{CH}_3$	0
2-Propyl formate	$\text{H}, i\text{-C}_3\text{H}_7$	0.300
n-Butyl formate	$\text{H}, n\text{-C}_4\text{H}_9$	0.360
2-Methyl-1-propyl formate	$\text{H}, i\text{-C}_4\text{H}_9$	0.365
1-Propyl formate	$\text{H}, n\text{-C}_3\text{H}_7$	0.375
Ethyl formate	$\text{H}, \text{C}_2\text{H}_5$	0.390
Methyl formate	$\text{H}, \text{CH}_3$	0.490
Ethyl benzoate	$\text{C}_6\text{H}_5, \text{C}_2\text{H}_5$	0.500
<i>Acids</i>		
Benzoic acid	$\text{C}_6\text{H}_5\text{COOH}$	0.600
Acetic acid	$\text{CH}_3\text{COOH}$	0
Propionic acid	$\text{CH}_3\text{CH}_2\text{COOH}$	-0.100
Butyric acid	$\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}$	-0.115
Valeric acid	$\text{CH}_3(\text{CH}_2)_3\text{COOH}$	-0.130
Lactic acid	$\text{CH}_3\text{CHOHCOOH}$	$\sigma^*\text{COOH} = -0.100$
		$\sigma^*\text{OH} = -0.100$
Malic acid	$\text{HOOC CHOCH}_2\text{COOH}$	$\sigma^*\text{COOH} = -0.200$
		$\sigma^*\text{OH} = -0.100$
Tartaric acid	$\text{HOOC}(\text{CHOH})_2\text{COOH}$	$\sigma^*\text{COOH} = -0.200$
		$\sigma^*\text{OH} = -0.200$
Citric acid	$\text{HOOC CH}_2\text{C}(\text{OH})\text{COOH} - \text{CH}_2(\text{COOH})$	$\sigma^*\text{COOH} = -0.420$
		$\sigma^*\text{OH} = -0.190$
<i>Sugars</i>		
D-Glucose	$\text{CH}_2\text{OH}(\text{CHOH})_4\text{CHO}$	$\sigma^*\text{CHO} = -0.133$
		$\sigma^*\text{OH} = -0.951$



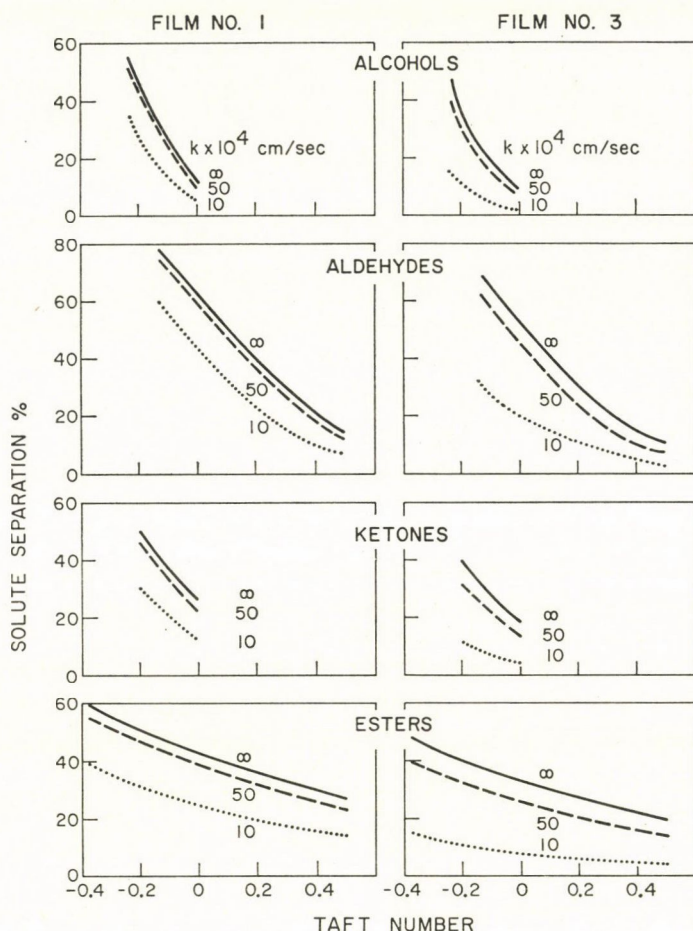


Fig. 1. Effect of Taft number on reverse osmosis separation of alcohols, aldehydes, ketones and esters. Film type, cellulose acetate, Batch 316; operating pressure, 250 psig

Table 4  
Polar functional constants ( $\rho^*$ ) for porous cellulose acetate membranes  
at 250 psig

Class of compounds	Range of $\sigma^*$ or $\Sigma\sigma^*$	$\rho^*$	Reference solute	
			Name	$\sigma^*$ or $\Sigma\sigma^*$
Alcohols	0 to -0.3	9.7		
	-0.3 to -0.68	5.8	1,3-Butanediol	-0.34
Aldehydes	-0.19 to 0.6	4.85	i-Butyraldehyde	-0.19
Ketones	-0.38 to 0	5.61	Acetone	0
Esters	-0.2 to 1.05	1.49	Methyl acetate	0

aration depends on (i) the porous structure of the membrane, (ii) the nature of the functional group, (iii) the Taft number for the substituent group and (iv) the mass transfer coefficient on the high pressure side of the membrane under the operating conditions. A smaller average pore size on the membrane surface results in higher solute separation; consequently, by decreasing the average pore size on the membrane surface, solute separation can be increased with respect to each solute. The total polar effect of the solute molecule includes contributions due to both the substituent and the functional groups in the molecule. Since Taft number represents the polar effect of the substituent group only, the total polar effect of the molecule is different for different functional groups at a given Taft number, and the effect of a change in Taft number on the change in the total polar effect of the molecule is also different for different functional groups. These differences naturally affect solute separation. For example, Fig. 1 shows that, for film 1 and  $k = \infty$ , solute separations are 12, 63, 26 and 43%, respectively, for alcohols, aldehydes, ketones and esters with a Taft number of zero; when the latter number is  $-0.1$ , the corresponding solute separations are 27, 75, 37 and 47%. Generally, a more negative Taft number results in higher solute separation. The variations in the mass transfer coefficient ( $k$ ) on the high pressure side of the membrane have a significant effect on solute separation; a lower mass transfer coefficient results in lower solute separation and the change becomes steep for  $k$  values less than  $50 \times 10^{-4}$  cm/sec.

The foregoing comments illustrate the factors affecting solute separation in reverse osmosis with respect to alcohols, aldehydes, ketones and esters in aqueous solutions using porous cellulose acetate membranes. Even though the above results are for single solute systems, the same principles may be expected to apply for mixed solute systems as well. The foregoing results thus illustrate the usefulness of the approach presented, and the need for more extensive work on the subject.

*2.1.5. Separation of acids.* The acids may be present as dissociated or undissociated species. The physicochemical criteria for the separation of the dissociated and undissociated species are the same as those of inorganic ions and alcohols, respectively (MATSUURA & SOURIRAJAN, 1971b); solute separation for the dissociated acid is due to electrostatic repulsion of ions, and that for the undissociated acid is due to the acidity (hydrogen bonding ability) of the molecule. While repulsion of ions always results in preferential sorption for water at the membrane-solution interface, and hence positive solute separation in reverse osmosis, either water or undissociated acid may be preferentially sorbed at the interface depending on the relative acidities of the respective molecules.

The dissociation constant  $K_a$ , conveniently expressed as  $pK_a = -\log K_a$ , is a quantitative measure of the acidity of the molecule. A decrease in the value



of  $pK_a$  represents an increase in the acidity of the molecule. On the basis of the discussion relating to the separation of alcohols, it is clear that an increase in the acidity of the solute molecule tends to increase its hydrogen bonding ability which, in turn, tends to decrease the preferential sorption of water and hence solute separation in reverse osmosis. On the other hand when the acidity of the molecule is high enough to stretch the OH bond to the point of rupture, then the molecule dissociates and exists in solution as ions which are subject to electrostatic repulsion in the vicinity of the membrane surface as discussed earlier. An analysis of the  $pK_a$  versus solute separation data for monocarboxylic acids shows (MATSUURA & SOURIRAJAN, 1971b) that the  $pK_a$  range 4 to 4.6 is particularly significant for monocarboxylic acids in aqueous solution. When  $pK_a$  is greater than 4.6, the acidity (hydrogen bonding ability) of the molecule is low enough to result in preferential sorption for water and hence positive solute separation in reverse osmosis; when  $pK_a$  is less than 4, the dissociation of the acid is high enough to result again in net positive solute separation in reverse osmosis. With respect to the undissociated species, a transition from repulsion for solute to attraction for solute at the membrane-solution interface occurs in the  $pK_a$  region 4 to 4.6. Preferential sorption for solute at the membrane-solution interface may (or may not) result in solute enrichment (negative solute separation) in the product solution; whether or not such enrichment occurs depends on the relative mobility of the adsorbed species under the operating conditions of the reverse osmosis experiment, as discussed in the literature for the case of *p*-chlorophenol (MATSUURA & SOURIRAJAN, 1972b).

The degree of dissociation and  $pK_a$  thus affect the reverse osmosis separation of acids. Unique relationships have been shown to exist between  $pK_a$  and Taft number (MATSUURA & SOURIRAJAN, 1971b; TAFT, 1956). Consequently the degree of dissociation and Taft number emerge as the governing physico-chemical criteria for the reverse osmosis separation of organic acids in aqueous solutions. These criteria can be used to study the reverse osmosis separation of fruit juice acids. This is illustrated in Figs. 2. and 3.

The major acids present in fruit juices are lactic, malic, tartaric and citric which are present in concentrations of 0.03 to 1.7% (Table 1); acetic, propionic, butyric, valeric and benzoic acids are also present in fruit juices as flavour compounds in extremely small concentrations. A set of experimental data on the separation of the above acids are given in Figs. 2 and 3 for two samples of cellulose acetate membranes (films 1 and 3) at an operating pressure of 250 psig. The solute concentrations used in feed were in the range 0.02 to 0.49% for the hydroxy-carboxylic acids, and 50 to 500 ppm for monocarboxylic acids, and the feed flow conditions used in reverse osmosis experiments corresponded to a mass transfer coefficient  $k$  of  $57 \times 10^{-4}$  cm/sec for 1 500 ppm NaCl—H<sub>2</sub>O feed solution. The solute separation data are expressed as functions of degree of dissociation and Taft number with respect to the acid molecule.

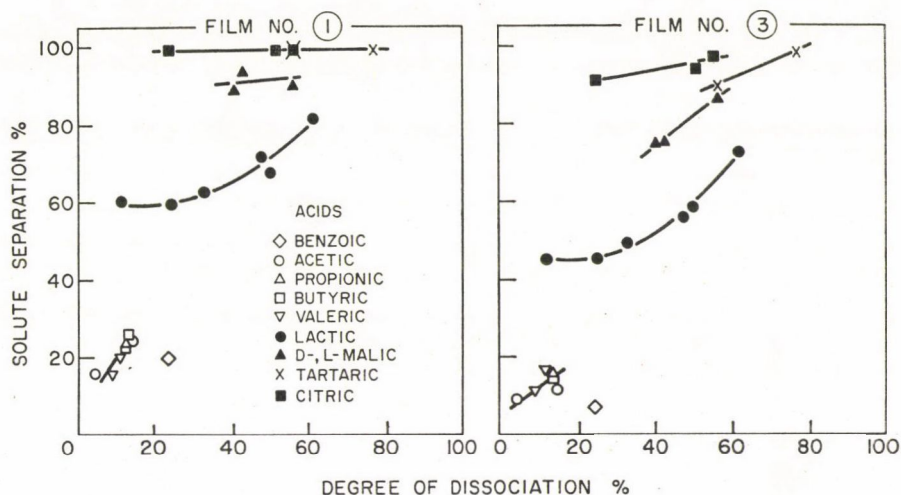


Fig. 2. Effect of degree of dissociation on reverse osmosis separation of acids. Film type, cellulose acetate, Batch 316; operating pressure, 250 psig

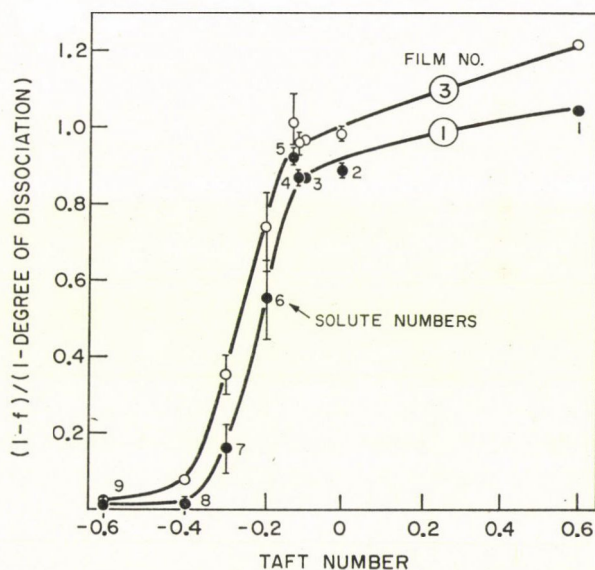


Fig. 3. Effect of Taft number on  $(1-f)/(1-d.d.)$  for acids. Film type, cellulose acetate, Batch 316; operating pressure, 250 psig; solute numbers, 1 benzoic acid; 2 acetic acid; 3 propionic acid; 4 butyric acid; 5 valeric acid; 6 lactic acid; 7 d-, l-malic acid; 8 tartaric acid; 9 citric acid

Fig. 2 shows that all the above acids can be separated in reverse osmosis to different extents under the experimental conditions used. The solute separation is generally in the order monohydroxy-tricarboxylic acid (citric) > dihydroxy-dicarboxylic acid (tartaric) > monohydroxy-dicarboxylic acid (malic)



>monohydroxy-monocarboxylic acid (lactic) >monocarboxylic acids (acetic, propionic, butyric, valeric and benzoic). The above order indicates that an increase in the number of polar functional groups in the solute molecule results in higher preferential sorption for water, and hence higher solute separation in reverse osmosis. Further, when the degree of dissociation of the acid molecule is increased, its separation is also increased in all cases.

The Taft numbers for all the above acids are given in Table 3. While each monocarboxylic acid has naturally a single Taft number, each hydroxy-carboxylic acid has two Taft numbers, one for each functional group. For purposes of correlation of reverse osmosis data in Fig. 3, the Taft number for each hydroxy-carboxylic acid is arbitrarily expressed as the sum of its Taft number for each functional group ( $\sigma_{\text{COOH}}^* + \sigma_{\text{OH}}^*$ ). [As pointed out earlier,  $\sigma^*$  values for different functional groups are not generally in the same scale with respect to their effect on solute separation in reverse osmosis. Consequently, such simple summation as expressed above is not generally valid. Appropriate techniques should be developed for expressing the effective Taft number for substituent groups in molecules containing different functional groups.]

Fig. 3 gives the correlation of Taft number versus the quantity  $(1-f)/(1-d.d.)$  where  $f$  represents fraction overall solute separation (*i.e.*, separation of both dissociated and undissociated species), and  $d.d.$  represents fraction degree of dissociation of solute in the feed solution. The correlation is valid for conditions in which  $d.d. \neq 1.0$ . The data cover the range of  $d.d.$  indicated in Fig. 2, and the quantity  $(1-f)/(1-d.d.)$  was found to be reasonably constant for the range of  $d.d.$  studied for each solute. There was some random scatter in the data as indicated in the correlation.

Fig. 3 shows that with each film, solute separation increases [*i.e.*,  $(1-f)/(1-d.d.) \rightarrow 0$ ] as the Taft number becomes more negative. The data are particularly interesting with respect to the effect of Taft number on the preferential sorption characteristic of the undissociated acid. When  $d.d. = 0$ ,  $f$  refers entirely to the undissociated species and values of 0 and 1.0 for the quantity  $(1-f)/(1-d.d.)$  correspond to 100% and 0% solute separation; a value of  $>1$  for the above quantity corresponds to negative solute separation, *i.e.*, solute enrichment in the product, indicating preferential sorption for solute at the membrane-solution interface. Referring to data for film 1, Fig. 3 shows that the undissociated citric acid ( $\Sigma\sigma^* = -0.61$ ) and tartaric acid ( $\Sigma\sigma^* = -0.40$ ) were almost completely separated, whereas undissociated benzoic acid ( $\sigma^* = 0.60$ ) was negatively separated and hence preferentially sorbed at the interface.

The foregoing results and discussion show that the degree of dissociation of the acids present, and the Taft number for the undissociated acids constitute the governing physicochemical criteria for the reverse osmosis separation of



fruit juice acids. The transition from preferential sorption for water to preferential sorption for undissociated acid seems to occur at a Taft number between 0 and 0.6; further experimental work can fix the transition point or range more precisely. When the undissociated solute is preferentially sorbed at the interface, both positive and negative solute separations can occur depending on the relative mobility of the adsorbed species under the experimental conditions as illustrated earlier for the case of *p*-chlorophenol (MATSUURA & SOURIRAJAN, 1972b).

*2.1.6. Separation of sugars.* Glucose, fructose and sucrose are the major sugars in fruit juices. Glucose has a Taft number of  $-0.951$  with respect to  $-\text{OH}$ , and  $-0.133$  with respect to  $-\text{CHO}$ . No method has yet been developed for estimating the Taft number for fructose and sucrose whose polyfunctional groups include carbonyl and glycoside linkages. It is, however, reasonable to consider that the effective Taft number for fructose is about as much as it is for glucose, and the effective Taft number for sucrose is about twice as much as it is for glucose. Thus the sugars in fruit juices have very high negative Taft numbers, which explains why sugars are relatively far more separated in reverse osmosis than all the other compounds listed in Table 3. This is illustrated by the solute separations of 98.4%, 99.2%, and  $\sim 100\%$ , respectively, for D-glucose, D-fructose and sucrose obtained with film 1 at 250 psig under experimental conditions comparable to those applicable for data given in Figs. 1, 2 and 3 for other solutes.

*2.1.7. Relative separation of fruit juice components during reverse osmosis concentration.* On the basis of the foregoing discussion, the Taft number criteria governing relative separation of alcohols, aldehydes, ketones, esters, (undissociated) acids and sugars during the reverse osmosis concentration of fruit juices using porous cellulose acetate membranes, may be stated explicitly as follows.

A relative decrease in Taft number represents a corresponding increase in the net basicity of the molecule. When the Taft number for solute is relatively less than the equivalent Taft number for water, the latter is preferentially sorbed at the membrane-solution interface. Each Taft number representing a solute corresponds to a definite critical pore diameter on the membrane surface, capable of giving practically complete solute separation and maximum water permeation through the membrane under the reverse osmosis operating conditions. A more negative Taft number for solute corresponds to a larger critical pore diameter on the membrane surface. If the pore diameter on the membrane surface is fixed so that it corresponds to the critical pore diameter for any particular Taft number, all solutes whose Taft numbers are higher will naturally permeate through the membrane pores to different extents under the reverse osmosis conditions. For example, if the pore size chosen on the membrane surface is the critical pore diameter corresponding to the Taft number for



glucose, one should expect that glucose, fructose and sucrose will be separated essentially completely in reverse osmosis, and all other solutes whose effective Taft numbers are more than that of glucose will permeate through the membranes to different extents. If the Taft number for solute is greater than that of water, solute will be preferentially sorbed on the membrane surface, under which conditions, solute separation in reverse osmosis could be positive, negative, or zero depending on operating conditions. If the Taft number for solute is identical to that of water, no solute separation is possible whatever be the porous structure of the membrane surface. Thus, the relative separation of fruit juice components during reverse osmosis concentration depends on the average pore size (pore size and pore size distribution) on the membrane surface, the Taft number of the fruit juice components relative to that of water and the operating conditions of the experiment.

*2.1.8. Nonpolar effect on solute separation.* The foregoing discussion is concerned with the polar effect of solute, as represented by its Taft number, on the preferential sorption of water. The extent of the latter should also be expected to be governed by the "nonpolar effect", or the hydrophobic character of the solute molecule, since the cellulose acetate membrane material has both hydrophilic and hydrophobic character. This subject is still under investigation, and hence no detailed discussion is possible at this time. It is, however, important to record here that the above "nonpolar effect" exists, and it has a governing significance with respect to reverse osmosis separation of solutes in aqueous solution using porous cellulose acetate membranes. No physico-chemical parameter has yet been chosen to represent effectively the nonpolar effect of a solute molecule. A few general observations, however, can be made. The membrane surface attracts a molecule which has a significant hydrophobic character. The result of this attraction is either to reduce the preferential sorption of water (and hence reduce solute separation in reverse osmosis), or to promote the preferential sorption of solute at the membrane-solution interface which, in turn, results in positive, negative, or zero solute separation in reverse osmosis. Solute molecules which do not contain any polar functional group, like benzene, may be expected to be preferentially sorbed on the membrane surface. Even solute molecules which have polar functional groups may be expected to have some hydrophobic character. Preliminary experiments show that molecules containing a straight chain involving 3 or more carbon atoms *not* associated with any polar functional group, exhibit significant nonpolar effect; with respect to such molecules, solute separation in reverse osmosis will be governed simultaneously by both the polar and nonpolar effects. This is illustrated by the experimental data reported earlier (MATSUURA & SOURIRAJAN, 1973) that solute separations for  $n\text{-C}_4$ ,  $\text{C}_6$  and  $\text{C}_7$  alcohols were lower than the separation obtained for  $n\text{-C}_3$  alcohol in spite of the more negative  $\Sigma\sigma^*$  values for the former alcohols. In view of the fact that many hydrocarbons,



higher alcohols and other compounds involving a chain of 3 or more carbon atoms *not* associated with any polar functional group, are present in fruit juices, the nonpolar effect is extremely significant with respect to the relative separation of fruit juice components in reverse osmosis, and hence the subject calls for detailed studies. It may be mentioned here that the data on relative solute separation given in Fig. 1 are on the basis of polar effect only.

*2.1.9. Effect of nature of solutes in fruit juices on product rate.* The water permeation rate through the membrane during fruit juice concentration by reverse osmosis is generally lower than what one would expect, for example, for an aqueous sodium chloride solution of the same osmotic pressure. There are several reasons for this.

First, the water permeation rate through the membrane is highly affected by the mass transfer coefficient  $k$  on the high pressure side of the membrane. The lower the value of  $k$ , the higher is the concentration polarization, and hence the product rate is lower. Under the same feed flow conditions, the values of  $k$  for sucrose-water and glucose-water feed solutions are far lower than the corresponding value of  $k$  for a comparable NaCl—H<sub>2</sub>O feed solution (SOURIRAJAN, 1970b; MATSUURA & SOURIRAJAN, 1971a). This is essentially because of the lower diffusivity of sugar in water. Further, fruit juices contain significant amounts of pectins which enhance the viscosity of the solutions and consequently reduce the mass transfer coefficients still further during the reverse osmosis operation. Thus the small values of  $k$  prevailing during the reverse osmosis concentration of fruit juices are mainly responsible for low product rates. Some data on the values of  $k$  obtained during the reverse osmosis processing of fruit juices with the apparatus used in this work are reported later in this paper.

There are two other equally important reasons for low product rates in fruit juice concentration by reverse osmosis using porous cellulose acetate membranes. The presence of alcohols of high acidity ( $\Delta v_s$ ) causes a densification of the porous structure of the membrane material as a result of induced intermolecular hydrogen bonding between the solute and the cellulose acetate molecule because of their proximity to each other. This densification increases resistance to fluid flow and hence reduces product rate as reported and discussed in the literature (MATSUURA & SOURIRAJAN, 1971b). Further, when the solute molecule is preferentially sorbed on the membrane surface, the former may exist partly or totally as an immobile layer which tends to block the pore area available for fluid flow and hence reduce the product rate. This possibility has been illustrated before with respect to p-chlorophenol (MATSUURA & SOURIRAJAN, 1972b), and it is again illustrated here in Fig. 4 with respect to benzene in aqueous solution. Since hydrocarbons like benzene are found in fruit juices (TRESSLER & JOSLYN, 1971), the data given in Fig. 4 are pertinent to the problem of fruit juice concentration.



Fig. 4 gives the performance data for two samples of cellulose acetate membranes (films **28** and **29**) of different surface porosities. In these experiments, the operating pressure was 500 psig, and benzene concentration in feed ranged from 20 to 555 ppm; the feed flow rate used was the same in both cases. Positive solute separation was obtained with each film. The solute separation remained essentially constant at 66% for film **28** and 50% for film **29** in the feed concentration range 20 to 400 ppm of benzene; when the concentration

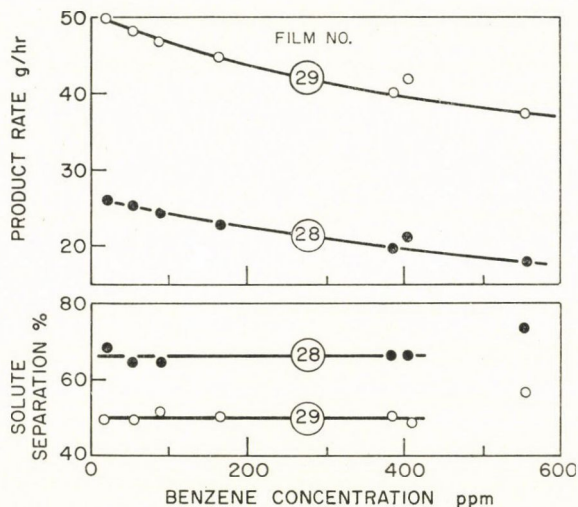


Fig. 4. Data on membrane performance for the reverse osmosis treatment of benzene in aqueous solutions. Film type, cellulose acetate, Batch 316; operating pressure, 250 psig

of the latter was increased to 555 ppm in feed, solute separation increased to 74% for film **28** and 56% for film **29**. With respect to product rate, even though the osmotic pressure effects were negligible because of the very low feed concentrations used, the product rates decreased significantly with increase in feed concentration. For example, the product rate decreased from 26 grams/hr to 18 grams/hr for film **28**, and 49.5 grams/hr to 37 grams/hr for film **29** when the concentration of benzene in feed increased from 20 to 555 ppm. These data illustrate the progressive increase in the concentration of solute in the preferentially sorbed region, and the decrease in pore area available for fluid flow on the membrane surface due to the blocking of pores by the relatively less mobile benzene molecules.

The decrease in product rates caused by the presence of high acidity or preferentially sorbed solutes referred to above, may be temporary or permanent depending on the concentration of such solutes in the feed solution and the time of contact of such solutes with the membrane surface under the reverse osmosis operating conditions. During the reverse osmosis concentration of



fruit juices, such reduction in product rates do occur. This is illustrated in Table 5 which gives data on the performance of different membranes in terms of their pure water permeation rate [PWP], product rate [PR], and solute separation with respect to 5 000 ppm NaCl—H<sub>2</sub>O feed solution before and after concentration of different fruit juices to twice their initial concentration. These experiments were carried out in the nonflow-type cell at an operating pressure of 1 000 psig. The fruit juice concentration process usually took about 2 to 10 hours. Table 5 shows the effect of apple juice, pineapple juice, orange juice, grapefruit juice and grape juice on the pore structure of the respective membranes used. The results show that in all the cases tested, the porous structure of the membrane was affected by contact with fruit juices as indicated by a decrease in [PWP] and [PR], and an increase in solute separation. From a practical point of view, these results indicate that it might be necessary to replace the membrane after every batchwise operation in the reverse osmosis concentration of fruit juices.

Table 5

*Data on membrane performance for the system sodium chloride—water before and after concentration of fruit juices*

Feed solution: 5 000 ppm NaCl—H<sub>2</sub>O.

Operating pressure: 1 000 psig.

Effective film area: 9.6 cm<sup>2</sup>.

Film number	Experiment	[PWP] grams/hr	[PR] grams/hr	Solute sepn. %
J1	Before apple juice concentration	84.7	69.2	93.5
	After apple juice concentration	75.6	—	95.1
J2	Before pineapple juice concentration	96.7	74.5	92.0
	After pineapple juice concentration	52.3	40.8	95.6
J3	Before orange juice concentration	75.4	60.9	95.0
	After orange juice concentration	64.6	56.3	96.1
J4	Before grapefruit juice concentration	107.6	89.4	84.1
	After grapefruit juice concentration	66.1	62.0	88.1
J5	Before grape juice concentration	123.7	102.0	85.7
	After grape juice concentration	92.5	82.0	91.5

#### 2.1.10. Solute recovery in fruit juice concentration by reverse osmosis.

On the basis of the physicochemical criteria discussed above for the reverse osmosis separation of fruit juice components, it is clear that by the choice of appropriate experimental conditions including membranes of different surface porosities, positive solute separation by reverse osmosis is possible with respect to all solutes which are positively or negatively adsorbed on the membrane surface. Since all solutes in fruit juices probably satisfy the latter condition



with respect to cellulose acetate membranes, solute recovery in fruit juice concentration by reverse osmosis can be accomplished to any practically desired extent by the repeated application of the technique for the treatment of both the fruit juices, and the membrane permeated fruit juice-waters. Hence, for the practical success of reverse osmosis for fruit juice concentration, it is suggested that the process be carried out in more than one stage. In the first stage, the major objective is naturally the almost complete ( $>99\%$ ) recovery of fruit juice sugars; in the subsequent stages, the objective could be the recovery of the other fruit juice components by the reverse osmosis treatment of membrane permeated fruit juice-waters obtained from the first stage operation, under appropriate experimental conditions.

## 2.2. Equations of reverse osmosis transport applicable to concentration of fruit juices

The need for transport equations arises from the need for determining parameters for specification of membranes and prediction of their performance in reverse osmosis. These equations are also needed to determine the osmotic pressure of fruit juices and their concentrates. Even though transport equations applicable for the reverse osmosis separation of single solute-aqueous solution systems have been well established, some modification of the form of these equations is necessary for their use in the processing of fruit juice solutions.

The Kimura—Sourirajan analysis of reverse osmosis experimental data, which is applicable to all levels of solute separation, gives rise to the following basic equations relating the pure water permeability constant  $A$  (in g mole  $\text{H}_2\text{O}/\text{cm}^2 \text{ sec atm}$ ), the transport of solvent water  $N_B$  (g mole  $\text{H}_2\text{O}/\text{cm}^2 \text{ sec}$ ), the solute transport parameter ( $D_{AM}/K\delta$ ) (cm/sec), and the mass transfer coefficient  $k$  (cm/sec) on the high pressure side of the membrane at any point (position or time) in the system under operating conditions of constant temperature and pressure.

$$A = \frac{[\text{PWP}]}{M_B \times S \times 3\,600 \times P}, \quad (5)$$

$$N_B = A[P - \pi(X_{A2}) + \pi(X_{A3})] \quad (6)$$

$$= \left( \frac{D_{AM}}{K\delta} \right) \left( \frac{1 - X_{A3}}{X_{A3}} \right) (c_2 X_{A2} - c_3 X_{A3}) \quad (7)$$

$$= kc_1(1 - X_{A3}) \ln \left( \frac{X_{A2} - X_{A3}}{X_{A1} - X_{A3}} \right). \quad (8)$$

The symbols used are listed and defined at the end of the paper. The above equations have been derived and discussed extensively in the literature (SOURIRAJAN, 1970b).

From the experimental pure water permeation rate [PWP] and product rate [PR] (grams/hr/S cm<sup>2</sup> of film area), and solute separation data, the values of  $A$ ,  $(D_{AM}/K\delta)$  for the solute, and  $k$  for the experimental conditions used, can be obtained from equations 5 to 8. At a given operating pressure and temperature, while  $A$  and  $(D_{AM}/K\delta)$  specify a membrane, data on all the three quantities  $A$ ,  $(D_{AM}/K\delta)$ , and  $k$  are needed to predict the performance of the membrane for different feed concentrations and feed flow conditions.

It may be noted that equations 6, 7 and 8 involve quantities for which a knowledge of the molecular weight of solute is needed. Since no definite value can be assigned to the molecular weight of solute in fruit juices, the above equations cannot be used directly to calculate  $(D_{AM}/K\delta)$  and  $k$  in the reverse osmosis processing of fruit juices. For the latter purpose, the set of equations 5 to 8 can be written in an analogous form as follows, in terms of quantities expressed in readily measurable weight units and carbon weight fractions:

$$A_{(wt)} = \frac{[\text{PWP}]}{S \times 3600 \times P}, \quad (9)$$

$$N_{B(wt)} = A_{(wt)}[P - \pi(X_{C2}) + \pi(X_{C3})] \quad (10)$$

$$= \left( \frac{D_{AM}}{K\delta} \right) \left( \frac{1 - X_{C3}}{X_{C3}} \right) [c_{2(wt)} X_{C2} - c_{3(wt)} X_{C3}] \quad (11)$$

$$= k c_{1(wt)}(1 - X_{C3}) \ln \left[ \frac{X_{C2} - X_{C3}}{X_{C1} - X_{C3}} \right], \quad (12)$$

where  $A_{(wt)}$ ,  $N_{B(wt)}$  and  $c_{(wt)}$  are in units of grams/cm<sup>2</sup> sec atm, grams/cm<sup>2</sup> sec and grams/cm<sup>3</sup> respectively, and  $X_C$  represents carbon weight fraction in solution (= weight of carbon in solution/total weight of solution). The subscripts 1, 2 and 3 in all cases represent the feed solution, concentrated boundary solution and the membrane permeated product solution, respectively. The relation between  $X_A$  (mole fraction of solute) and  $X_C$  (carbon weight fraction in solution) is

$$X_C = \frac{X_A M_C}{M_A X_A + M_B(1 - X_A)},$$

where  $M_A$ ,  $M_B$  and  $M_C$  represent respectively molecular weight of solute, molecular weight of water and molecular weight of solute with respect to carbon only.



In view of the form of above equation, it is not obvious that the values of  $(D_{AM}/K\delta)$  and  $k$  calculated on the basis of equations 5 to 8 will be identical to those calculated on the basis of equations 9 to 12. This was checked with a set of reverse osmosis data obtained with aqueous sucrose feed solutions using a membrane (film **J12**) which gave  $>99\%$  solute separation under the operating conditions used. These experiments were conducted in the nonflow-type cell at 1 000 psig. From the experimental [PWP], [PR] and solute separation data, the values of  $(D_{AM}/K\delta)$  and  $k$  were calculated by both the methods using equations 5 to 8 and equations 9 to 12. The results, given in Table 6, showed that both methods of calculation gave identical values for  $(D_{AM}/K\delta)$  and  $k$  establishing thereby the equivalence of the two sets of equations for the reverse osmosis treatment of sugar solutions. Consequently, for the reverse osmosis processing of fruit juices, the basic transport equations 5 to 8 can be used in their analogous form represented by equations 9 to 12.

Table 6

*Comparison of data on  $(D_{AM}/K\delta)$  and  $k$  for the system sucrose-water calculated on molar basis and on carbon weight basis*

Film number: **J12**.

Operating pressure: 1 000 psig.

Sucrose concn. in feed		$(D_{AM}/K\delta) \times 10^5$ cm/sec		$k \times 10^4$ cm/sec	
Mole fraction $X_A \times 10^3$	Carbon wt. fraction $X_C \times 10^2$	Molar basis	Carbon wt. basis	Molar basis	Carbon wt. basis
3.6	2.7	0.546	0.546	13.6	13.6
7.2	5.07	0.513	0.512	13.4	13.4
10.7	7.18	0.402	0.400	12.8	12.8
14.2	9.05	0.339	0.336	12.2	12.0
17.7	10.72	0.075	0.075	9.8	9.6

### 2.3. Determination of osmotic pressure of fruit juices and their concentrates

The osmotic pressure of a fruit juice or its concentrate has often been given as the pressure estimated for zero product rate obtained from extrapolation of experimental pressure versus product rate data (MERSON & MORGAN, 1968). This technique neglects the errors caused by the effects of (i) concentration polarization on the membrane surface under the experimental conditions, (ii) concentration of solutes in the product water and (iii) membrane compaction effects at high operating pressures. Even though these effects may be negligible in particular cases, a technique which inherently neglects these effects cannot be generally valid. A more acceptable experimental technique



which does not neglect any of the above effects, is suggested and illustrated below for the determination of osmotic pressure of fruit juices and their concentrates.

In this technique the solute concentrations in fruit juice solutions are expressed in terms of their carbon content as carbon weight fraction  $X_C$ . The porous structure of the membranes used in this technique is similar to those normally chosen for fruit juice concentration *i.e.*, those capable of giving  $\sim 99\%$  solute separation at operating pressures of 1 000 psig or higher.

In addition to the natural fruit juice, fruit juice solutions of several other concentrations for which osmotic pressure data are needed, are first prepared. This is done by concentrating the fruit juice in a preliminary reverse osmosis experiment at a high operating pressure ( $\sim 1\,000$  psig) using a membrane capable of giving  $>99\%$  solute separation with respect to total carbon. The membrane permeated fruit juice water obtained in the above concentration process is carefully collected for re-use. Parts of the concentrated fruit juice are then diluted to the required extent by the fruit juice water collected during the concentration process, to obtain samples of fruit juice solutions of different concentrations for subsequent osmotic pressure determinations.

The experiments for osmotic pressure determinations are carried out in the nonflow-type cell. For each fruit juice solution two experiments are carried out, each at a different pressure. Each experiment involves the determination of [PWP], [PR] and solute separation at the given operating pressure. The first experiment (called the "low-pressure experiment") is at a pressure only slightly higher than the osmotic pressure of the fruit juice solution under study; at the pressure used for this experiment, the product rate is small, and hence the concentration polarization also is small. The second experiment (called the "high-pressure experiment") is at a pressure (600 or 1 000 psig in this work) much higher than the osmotic pressure of the fruit juice solution; under the conditions of the latter experiment, both product rate and concentration polarization are much higher than those obtained at the low-pressure experiment. Both experiments are of the short-run type, *i.e.*, the net amount of product removed is small, compared to the amount of feed in the cell so that there is no significant change in concentration in the feed solution during the experiment. For each fruit juice solution, the density of feed  $c_{1(wf)}$ , carbon weight fraction in feed  $X_{C1}$ , carbon weight fraction in product  $X_{C3}$ , and product water flux  $N_{B(wf)}$ , for both the low-pressure and high-pressure experiments, along with the corresponding values of pure water permeability constants  $A_{(wf)}$ , are known. From similar data for several concentrations ( $X_C$ ) of fruit juice solutions, the correlation between  $X_C$  and osmotic pressure is established by a process of successive approximation as described below.



The following forms of equations 10 and 12 are used in these calculations:

$$\pi(X_{C2}) = P - \frac{N_{B(wt)}}{A_{(wt)}} + \pi(X_{C3}) \quad (13)$$

$$k = N_{B(wt)}/c_{1(wt)}(1 - X_{C3}) \ln \left[ \frac{X_{C2} - X_{C3}}{X_{C1} - X_{C3}} \right] \quad (14)$$

$$X_{C2} = X_{C3} + (X_{C1} - X_{C3}) \exp \left[ \frac{N_{B(wt)}}{k c_{1(wt)}(1 - X_{C3})} \right] \quad (15)$$

The calculation procedure involves the following steps:

*Step 1.* Consider low-pressure data for all feed concentrations. As a first approximation assume  $X_{C2} = X_{C1}$ , and  $X_{C3} = 0$ ; then equation 13 approximates to

$$\pi(X_{C1}) = P - \frac{N_{B(wt)}}{A_{(wt)}} \quad (16)$$

Apply equation 16 to all low-pressure data. Since all quantities on the right hand side of equation 16 are known, calculate  $\pi(X_{C1})$  for the different values of  $X_{C1}$ . Plot the values of  $X_{C1}$  versus  $\pi(X_{C1})$ . Let this correlation of  $X_C$  versus  $\pi(X_C)$  be called the "first-approximation".

*Step 2.* Consider high-pressure data for all feed concentrations. For each case find  $\pi(X_{C3})$  from first approximation. Using this value and equation 13, calculate  $\pi(X_{C2})$ . Using the first-approximation again, find  $X_{C2}$  corresponding to  $\pi(X_{C2})$ . Using this value of  $X_{C2}$  and equation 14, calculate  $k$ .

*Step 3.* Again consider low-pressure data for all feed concentrations. For each case, calculate  $X_{C2}$  from equation 15 using the value of  $k$  obtained in Step 2 above. Also, for each case, calculate  $\pi(X_{C2})$  using equation 13. Thus a new set of  $X_{C2}$  and  $\pi(X_{C2})$  data are obtained. Plot these data as before, and let this correlation of  $X_C$  versus  $\pi(X_C)$  be called the "second-approximation".

*Step 4.* Repeat steps 2 and 3 above using the second-approximation, and generate a new  $X_C$  versus  $\pi(X_C)$  correlation which may be called the "third-approximation".

*Step 5.* Repeat the above procedure, generating each time a new  $X_C$  versus  $\pi(X_C)$  correlation. Let these correlations be called successively as the 4th, 5th, . . .  $n$ th, and  $(n+1)$ th approximation. When the  $n$ th and  $(n+1)$ th approximations become essentially identical, the latter approximation may be con-



sidered as the valid correlation between  $X_C$  and  $\pi(X_C)$ , which establishes the osmotic pressure of the fruit juice solution as a function of its carbon weight fraction.

The applicability of the above technique was first tested for the system sucrose-water in the feed concentration range 6.4 to 25.5 weight % sucrose. The final results obtained are shown in Fig. 5 in which the points represent the experimental data and the solid line represents literature data (SOURIRAJAN,

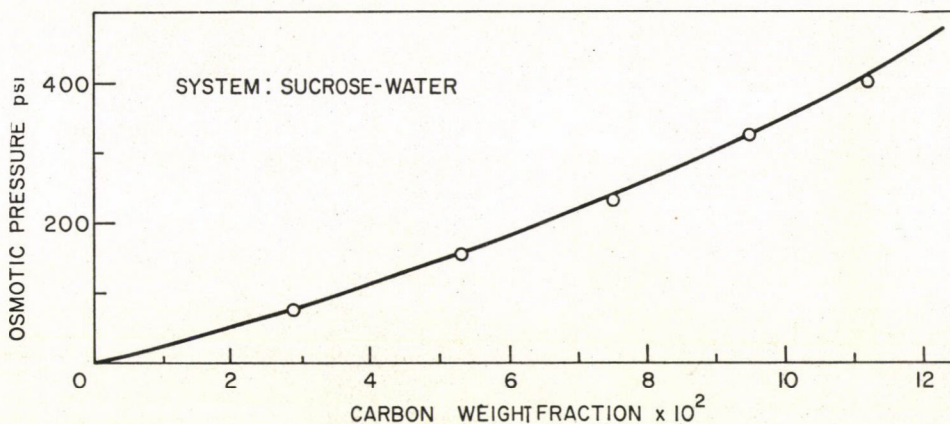


Fig. 5. Osmotic pressure of aqueous sucrose solutions.

— Literature data; ○ Experimental data

1970e) calculated on the basis of the activity of water in aqueous sucrose solutions. The excellent agreement between the experimental and literature values established the validity of the technique outlined above for the determination of osmotic pressures.

Following the above technique, the osmotic pressures of different concentrations of apple juice, pineapple juice, orange juice, grapefruit juice, grape juice and tomato juice solutions were experimentally determined. Three approximations were usually sufficient to arrive at the valid  $X_C$  versus  $\pi(X_C)$  correlation. This is illustrated in Fig. 6 which shows that, for the grape juice solutions the second and third-approximations were essentially identical. Fig. 7 gives the final results obtained for the osmotic pressures of all the fruit juice solutions studied as a function of their carbon weight fractions. Literature data (SOURIRAJAN, 1970e; MATSUURA & SOURIRAJAN, 1971a) for aqueous sucrose and aqueous glucose solutions are also shown in Fig. 7 for comparison.

Several aspects of the data presented in Fig. 7 are interesting. For the same carbon content, the values obtained for the osmotic pressures of tomato juice solutions were much higher than those obtained for the other fruit juice



solutions studied. This is believed to be essentially due to the presence of salt (sodium chloride) present in the commercial tomato juices used in this work. Both for their own sake, and for comparison with other fruit juice solutions, osmotic pressure determinations should be made for unsalted tomato juice solutions. With respect to the other fruit juice solutions studied, for the same

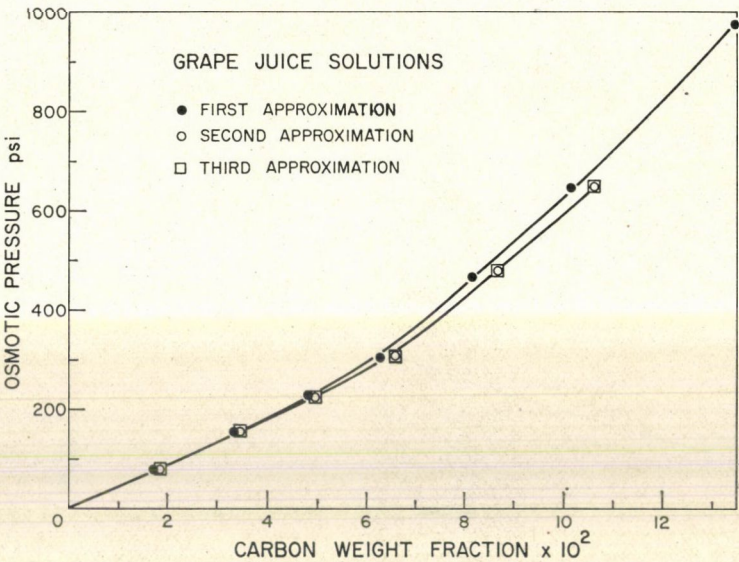


Fig. 6. Osmotic pressure of grape juice solutions

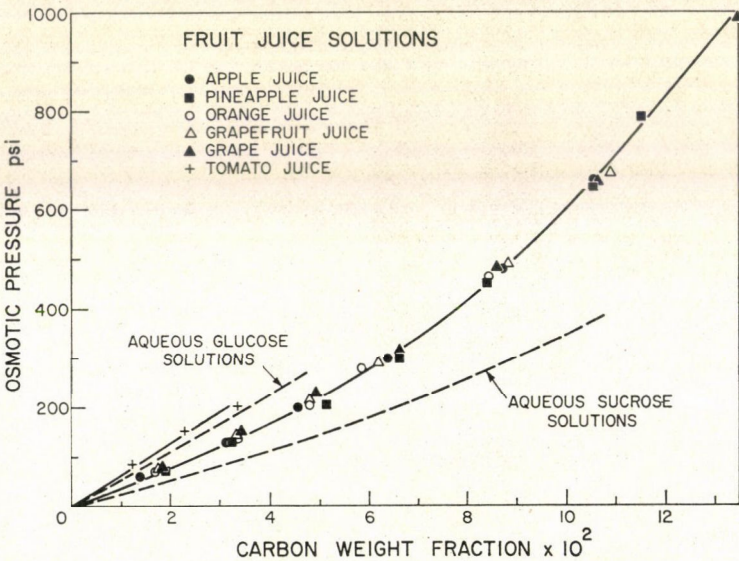


Fig. 7. Osmotic pressure of different fruit juice solutions



carbon content, the osmotic pressure of aqueous glucose solution is higher and that of aqueous sucrose solution is lower than the osmotic pressure of the corresponding fruit juice solution. This result indicates that the sugars in fruit juices constitute essentially a mixture of mono- and disaccharides. Further, the  $X_C$  versus osmotic pressure correlation is essentially identical for all the apple juice, pineapple juice, orange juice, grapefruit juice and grape juice solutions studied; this means that the osmotic pressure of all the above fruit juice solutions may be expressed by a single relationship in terms of carbon weight fraction in solution.

If it can be assumed that the osmotic pressure  $\pi$  of the fruit juice solution is proportional to the mole fraction  $X_A$  of an equivalent single solute whose molecular weight is  $M_A$  and whose molecular weight with respect to carbon is  $M_C$ , then the above assumption can be expressed as

$$\pi = BX_A, \quad (17)$$

where  $B$  is a proportionality constant. Using equation 13, equation 17 can be rewritten as

$$\frac{\pi}{X_C} = \frac{M_A - M_B}{M_C} \pi + \frac{M_B}{M_C} B, \quad (18)$$

which means that the plot of  $\pi$  versus  $\pi/X_C$  is a straight line. The above plot for all the fruit juice solutions studied (except the tomato juice solutions) is illustrated in Fig. 8 which shows that equation 18 and hence the assumption expressed by equation 17 are practically valid at least up to an osmotic pressure of 700 psi.

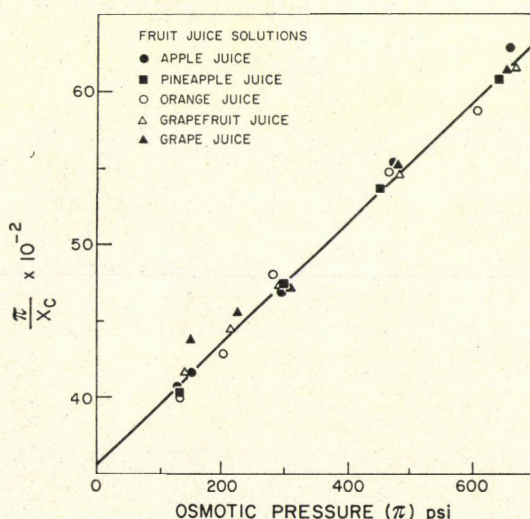


Fig. 8. Osmotic pressure versus (osmotic pressure/carbon weight fraction) correlation for different fruit juice solutions



The equation of the best straight line representing all the data plotted in Fig. 8 can be given as

$$\frac{\pi}{X_C} = 3.94 \pi + 3560 \quad (19)$$

from which  $\pi$  (in psi) can be given as

$$\pi = \frac{3560 X_C}{1 - 3.94 X_C} \quad (20)$$

Therefore, for practical purposes, equation 20 may be considered as a working relationship applicable for the determination of osmotic pressures of apple juice, pineapple juice, orange juice, grapefruit juice, grape juice and their respective concentrates.

#### 2.4. Some aspects of reverse osmosis process design for fruit juice concentration

**2.4.1. Basic concepts.** The concepts of membrane and system specifications, and predictability of membrane and system performance are fundamental to the engineering science of reverse osmosis process design. These concepts are extensively discussed in the literature with particular reference to saline water conversion and water treatment applications (SOURIRAJAN, 1970c; OHYA & SOURIRAJAN, 1971).

Every reverse osmosis membrane whose performance data are of interest must be properly specified to serve as a basis for process design. Descriptions of membranes by words like "tight" and "open" (so common in reverse osmosis literature) do not constitute any precise specification of a reverse osmosis membrane. On the other hand, a membrane is precisely and completely specified in terms of its pure water permeability constant  $A$ , and solute transport parameter ( $D_{AM}/K\delta$ ) for a reference solute such as sodium chloride, at a given operating pressure and temperature. Such specifications for all the membranes used in this work are given in Table 1.

In any reverse osmosis process, the membrane-solution-operating system may be specified by three fundamental nondimensional parameters —  $\gamma$ ,  $\Theta$ , and  $\lambda$  — defined as follows:

$$\gamma = \frac{\text{osmotic pressure of initial feed solution}}{\text{operating pressure}} = \frac{BX_{A1}^0}{P} \quad (21)$$

$$\Theta = \frac{\text{solute transport parameter}}{\text{pure water permeation velocity}} = \frac{(D_{AM}/K\delta)}{v_w^*} \quad (22)$$

$$\lambda = \frac{\text{mass transfer coefficient on the high pressure side of membrane}}{\text{solute transport parameter}} = \frac{k}{(D_{AM}/K\delta)} \quad (23)$$



The quantity  $v_w^*$  is obtained from relation

$$v_w^* = \frac{AP}{c}, \quad (24)$$

where  $c$  is the molar density of pure water.

The possible specification of membranes with respect to fruit juice solutes, and the application of reverse osmosis system analysis for the fruit juice concentration process are of practical interest.

In the reverse osmosis processing of fruit juices, the quantities  $A$  (as  $A_{(wt)}$ ),  $(D_{AM}/K\delta)$  and  $k$  can be obtained from equations 9 to 12 using the experimental data on [PWP], [PR], and solute separation (equation 2), together with a knowledge of the osmotic pressure of the fruit juice solution and its concentrates (equation 20). Using the above quantities, the parameters of system specification,  $\gamma$ ,  $\theta$  and  $\lambda$  for the fruit juice concentration process can be calculated from equations 21 to 24. The performance of the reverse osmosis system so specified can be predicted by the system analysis of OHYA and SOURIRAJAN (1971), provided the following conditions are satisfied:

1. osmotic pressure of solution is proportional to mole fraction of solute;
2. solute concentration in product is small compared to that of water;
3. longitudinal diffusion of solute is negligible in the process using a flow-type cell apparatus;
4.  $(D_{AM}/K\delta)$  is independent of feed concentration;
5. molar density of solution is constant, and it is essentially that of pure water;
6. membrane pore structure does not change during the process.

Condition 1 is satisfied on the basis of osmotic pressure data given in Figs. 7 and 8. Condition 2 is satisfied because of high solute separations obtained (in terms of total carbon content) in the concentration process. Condition 3 may be assumed valid at high feed flow rates. Regarding condition 4, some data on the effect of feed concentration on  $(D_{AM}/K\delta)$  for fruit juice solutes at 600 psig with membranes which are of practical interest for fruit juice concentration, are given in Table 7. The solute separation obtained with respect to total carbon content was  $>99\%$  in all the above cases. Table 7 shows that  $(D_{AM}/K\delta)$  for fruit juice solute is either independent of feed concentration (as in the case of orange juice and grapefruit juice solutions), or has a tendency to decrease with increase in feed concentration as in the case of apple juice, pineapple juice and grape juice solutions. The behaviour of the latter solutions seems to be similar to that of sucrose solutions (SOURIRAJAN, 1970b). In all cases, however, the actual values of  $(D_{AM}/K\delta)$  obtained at the different feed concentrations were extremely small,  $\sim 1 \times 10^{-5}$  cm/sec or less; under such conditions, the



effect of change in  $(D_{AM}/K\delta)$  on solute separation and product rate is negligible as illustrated in the literature for the case of glycerol-water system. Consequently, one may conclude that, whether  $(D_{AM}/K\delta)$  for fruit juice solute is independent of feed concentration or decreases with increase in feed concentration, when the absolute value of  $(D_{AM}/K\delta)$  is very small,  $\sim 1 \times 10^{-5}$  cm/sec or less — which is the case under the operating conditions of fruit juice concentration — it is reasonable to assume that condition 4 is practically satisfied for purposes of analysis. The situation regarding conditions 5 and 6 is different. The molar density of pure water may be considerably different from that of fruit juices and/or of their concentrates. It is known that the pore structure of the membrane surface is affected by continued contact with fruit juice solutions. Consequently, conditions 5 and 6 are not generally satisfied in the reverse osmosis concentration of fruit juices, which may make the predictions of Ohya-Sourirajan equations inaccurate. However, for the purpose of specifying the reverse osmosis system for fruit juice concentration, the pure water permeability constant and the molar density of fruit juice solutions have to be expressed in weight units. If the system specifications are based on average values of  $A_{(wt)}$  and  $c_{(wt)}$ , the errors arising from the assumptions of conditions 5 and 6 may be partly compensated, and the predictions of Ohya-Sourirajan equations may still be valid at least for parametric studies. This possibility is illustrated below with particular reference to apple juice concentration. More extensive work with a wide variety of fruit juice solutions is called for to explore the above possibility in all its aspects.

The feed flow condition in the apparatus during reverse osmosis operation is best expressed in terms of the applicable mass transfer coefficient  $k$  on the high pressure side of the membrane under the experimental conditions. It may be pointed out here that an expression of feed flow condition in the reverse osmosis apparatus in terms of Reynold's number is by itself not useful unless such data can be translated in terms of  $k$ . The value of  $k$  enters system specification in the parameter  $\lambda$  which is more usefully expressed in the form  $\lambda\Theta (= k/v_w^*)$ . An important parametric study in process design is the effect of  $\lambda\Theta$  on the  $\Delta$  versus  $\tau$  or  $X$  correlation which expresses the effect of mass transfer coefficient  $k$  on membrane area or processing time requirements for a given product water recovery with a given membrane in a reverse osmosis apparatus. The quantities  $\Delta$ ,  $\tau$  and  $X$  are defined as follows:

$$\Delta = 1 - \frac{V_1}{V_1^0} \text{ (batch operation)} \quad (26)$$

$$= 1 - \frac{\bar{u}}{u^0} \text{ (continuous operation)} \quad (27)$$

$$\tau = \frac{S v_w^* t}{V_1^0} \text{ (batch operation)} \quad (28)$$



$$X = \frac{v_u^* x}{\bar{u}^0 h} \text{ (continuous operation).} \quad (29)$$

The quantities  $\tau$  and  $X$  are numerically identical when  $1/h = S/V_1^0$  and  $x/\bar{u}^0 = t$ .

*2.4.2. Some experimental and analytical results.* The mass transfer coefficient  $k$  is essentially a function of feed concentration and feed flow rate in the flow-type apparatus and the degree of turbulence obtained in the nonflow-type apparatus. Fig. 9 gives the effect of feed concentration on  $k$  obtained in the nonflow-type apparatus used in this work. The results show that the values of  $k$  obtained in the apparatus were generally low and they were essentially the same for all the juice solutions studied except the tomato juice solutions. In the range of concentration studied, the average value of  $k$  was  $4.5 \times 10^{-4}$  cm/sec for tomato juice solutions, and  $\sim 10 \times 10^{-4}$  cm/sec for all the other fruit juice solutions. Under the same conditions, the average value of  $k$  obtained for aqueous sucrose solutions was  $\sim 12.5 \times 10^{-4}$  cm/sec. A lower value of  $k$  for

Table 7

*Effect of feed concentration on  $(D_{AM}/K\delta)$  for fruit juice solutes at 600 psig*

Film number	Feed solution	Carbon content in feed solution ppm	$(D_{AM}/K\delta) \times 10^5$ cm/sec
J7	Apple juice	29 900	0.81
		43 800	0.84
		61 900	0.66
		84 800	0.36
J8	Pineapple juice	29 800	0.64
		47 300	0.43
		62 200	0.24
		80 400	0.35
J9	Orange juice	30 800	1.32
		45 000	0.97
		80 200	1.18
J10	Grapefruit juice	31 700	0.66
		45 900	0.35
		58 500	0.77
		86 900	0.43
J11	Grape juice	33 300	1.12
		48 100	0.63
		62 700	0.39
		81 500	0.69

Experiments carried out in nonflow-type cell.



the fruit juice solutions compared to that for aqueous sucrose solutions of the same carbon content probably indicates the effect of increased viscosity of the former solutions. Fig. 10 gives the comparative values of  $k$  obtained in the flow-type cell for 3 500 ppm NaCl-H<sub>2</sub>O and apple juice solutions at the same feed flow rates. These data show that the values of  $k$  obtainable with apple juice solutions are far lower than those obtainable with aqueous sodium chloride solutions at the same feed flow rates.

A set of experiments on the reverse osmosis concentration of fruit juices was carried out at 1 000 psig in the nonflow-type cell. The quantity of product

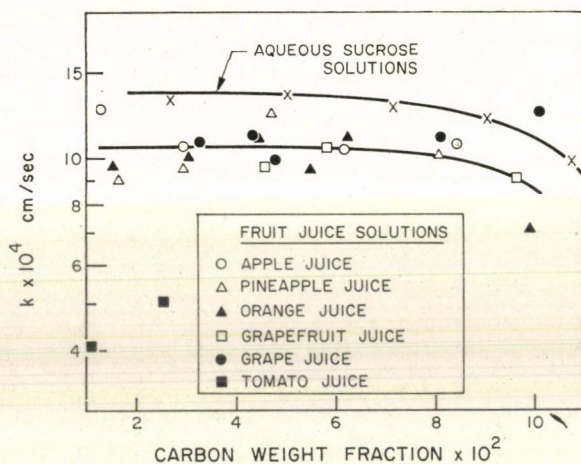


Fig. 9. Mass transfer coefficients obtained during reverse osmosis treatment of fruit juice solutions in the nonflow-type cell used. Film type, cellulose acetate, Batch 316; operating pressure, 1 000 psig

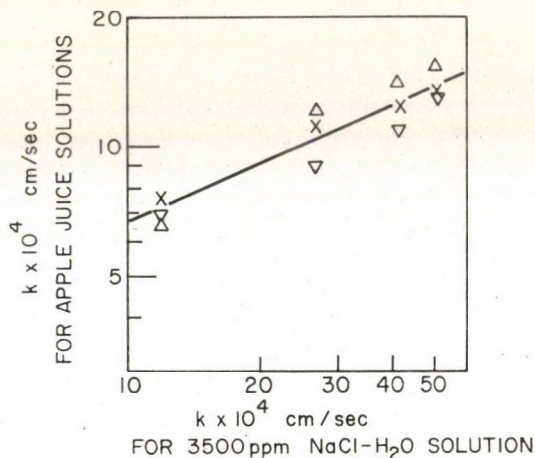


Fig. 10. Relative mass transfer coefficients obtained in the flowtype cell used for apple juice and aqueous sodium chloride feed solutions. Film type, cellulose acetate, Batch 316; operating pressure, 1 000 psig; concentration of apple juice feed solutions:  $\times$  14 800 ppm carbon;  $\Delta$  41 700 ppm carbon;  $\nabla$  96 500 ppm carbon

water removed in each case was 50% or more of the initial feed solution on a volume basis. The overall solute separation in terms of total carbon content in the concentration process was >99% in all cases. Consequently, the solute concentration in the fruit juice concentrate was about twice that in the initial feed. The data relating to these experiments are given in Tables 8 and 9. Two kinds of analytical results which are of interest in process design can be obtained from the above data. These results are illustrated below.

Table 8 gives the specifications of a film used for apple juice concentration. The value of  $A_{(wt)}$  given is the average of the values obtained before and after the concentration experiment. The density of the fruit juice solution given is the average of the values obtained for the solutions involved. The above average values were used to calculate  $v_w^*$ . The value of  $(D_{AM}/K\delta)$  given was obtained in a short-run experiment with apple juice feed with the above membrane; in the latter experiment, the mass transfer coefficient obtained was  $10 \times 10^{-4}$  cm/sec which was assumed constant for the concentration process. Using the above experimental data, the system specification for the concentration process could be given as  $\gamma = 0.188$ ,  $\Theta = 0.001$ , and  $\lambda\Theta = 0.45$ . The  $\Delta$  versus  $\tau$  or  $X$  correlation obtained from the Ohya-Sourirajan analysis corresponding to the above data on system specification is given in Fig. 11 in which the actual experimental data obtained are also plotted. The good agreement between the experimental and analytical results indicates the validity of the results of system analysis discussed above for apple juice concentration. The analytical results obtained on the effect of  $\lambda\Theta$  on the  $\Delta$  versus  $\tau$  or

Table 8

*Concentration of apple juice*Film number **J13**.

<i>Operating conditions</i>	
Operating pressure, atm	68.0
Mass transfer coefficient $k$ , cm/sec	$10.0 \times 10^{-4}$
<i>Properties of feed solution</i>	
Carbon content in feed, ppm	43 800
Osmotic pressure of feed, atm	12.82
Average density, grams/cm <sup>3</sup>	1.051
<i>Film specification</i>	
Pure water permeability constant, $A_{(wt)}$ , grams H <sub>2</sub> O/cm <sup>2</sup> sec atm	$34.24 \times 10^{-6}$
Solute transport parameter, $(D_{AM}/K\delta)$ , cm/sec	$0.2 \times 10^{-5}$
<i>System specification</i>	
$\gamma$	0.188
$\Theta$	0.001
$\lambda\Theta$	0.45



$X$  correlation are also plotted in Fig. 11. The latter results give a quantitative illustration of the reductions obtainable in the parameter  $\tau$  or  $X$  for a given value of  $\Delta$  by increasing the mass transfer coefficient in the operating system. For example, for  $\Delta = 0.5$ , the values of  $\tau$  or  $X$  are 1.3, 1.22, 0.9, 0.78, 0.71 and 0.68 corresponding to  $\lambda\theta$  values of 0.45, 0.5, 1.0, 2.0, 5.0, and  $\infty$ , respectively. These results illustrate the need for specifying and increasing the

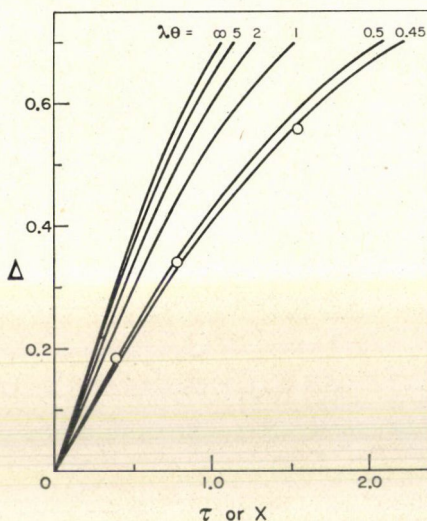


Fig. 11. Some results of system analysis for apple juice concentration by reverse osmosis. System specifications,  $\gamma = 0.188$ ,  $\theta = 0.001$ , and  $\lambda\theta = 0.45, 0.5, 1, 2, 5$ , and  $\infty$

$\lambda\theta$  values in a practical concentration process. These results also indicate that  $\lambda\theta$  values in the range 1 to 2 would probably be most desirable in practical fruit juice concentration systems. The system specifications and performance data included in the Ohya-Sourirajan tables (OHYA & SOURIRAJAN, 1971) may be used for similar parametric studies on fruit juice concentration systems.

For a batch concentration process, the quantity called "the processing capacity of the membrane for solute concentration" is a useful design parameter. The above quantity is defined (SOURIRAJAN, 1970d) as the volume of charge (feed solution) that 1 square foot of film surface can handle per day,  $(V)_i/St$ , in a batch concentration process under the specified experimental conditions. This quantity can be calculated from the following relation derived in the literature (SOURIRAJAN, 1970d):

$$\frac{(V)_i}{St} = q_{av} \left[ \frac{\alpha_v}{\alpha_v(\rho_1)_i - (\rho_1)_f} \right], \quad (30)$$



where  $q_{av}$  is the average product water flux (in weight units per unit area per unit time) during the concentration process,  $\alpha_v$  is the volume ratio of the feed with respect to the concentrate,  $(V)_i/(V)_f$ , and  $(\rho_1)_i$  and  $(\rho_1)_f$  are the densities of the feed and concentrate, respectively. Table 9 gives the processing capacities (in gallons/day ft<sup>2</sup>) of the films used in this work for the concentration of different fruit juices studied to give an  $\alpha_v$  value of 2. These data show that for the type of apparatus and films used in this work, the processing capacity was highest (31.4 gal/day ft<sup>2</sup>) for apple juice concentration and lowest (5.9 gal/day ft<sup>2</sup>) for orange juice concentration at the operating pressure of 1 000 psig. In view of the high economic value of the concentrated fruit juices, the above processing capacities are probably sufficiently high to be of practical interest in all cases. With a better apparatus capable of giving a higher mass transfer coefficient on the high pressure side of the membrane during the concentration process, much higher processing capacities can be obtained in each case. The data in Table 9 show that the process is particularly promising for the concentration of apple juice even under the experimental conditions used in this work.

Table 9  
*Concentration of fruit juice*

Experimental details	Apple juice	Pineapple juice	Orange juice	Grapefruit juice	Grape juice	Tomato juice
Film number	<i>J1</i>	<i>J2</i>	<i>J3</i>	<i>J4</i>	<i>J5</i>	<i>J6</i>
Operating pressure, psig	1 000	1 000	1 000	1 000	1 000	1 000
<i>Feed</i>						
Carbon weight fraction $\times 10^2$	4.38	5.30	5.24	5.38	5.98	2.91
Solid content, weight %	10.75	11.45	10.35	13.90	15.40	5.85
Density, gram/cm <sup>3</sup>	1.038	1.046	1.042	1.052	1.063	1.011
pH	3.9	3.7	4.2	4.2	3.2	4.7
<i>Product</i>						
Carbon weight fraction $\times 10^2$	0.0720	0.0385	0.0552	0.0750	0.0740	0.0440
<i>Concentrate</i>						
Carbon weight fraction $\times 10^2$	8.51	9.40	10.41	9.50	11.03	5.92
Solid content, weight %	19.35	21.65	22.45	25.25	25.70	12.85
Density, gram/cm <sup>3</sup>	1.074	1.089	1.081	1.105	1.107	1.100
Total carbon retained, %	99.2	99.6	99.5	99.3	99.5	99.2
Feed volume/concentrate volume	2	2	2	2	2	2
Av. product rate, grams/hour*	25.6	5.6	4.8	7.7	13.1	10.3
Processing capacity of film gal/day ft <sup>2</sup>	31.4	6.9	5.9	9.5	15.8	13.8

\* Film area = 9.6 cm<sup>2</sup>.



### 3. Conclusions

Reverse osmosis is a new and challenging process for the concentration of fruit juices. A fundamental approach to the science and engineering of reverse osmosis is necessary to realize the immense potentialities of this process for technical and economic success. This paper offers a basis for such an approach with respect to the application of reverse osmosis for fruit juice concentration.

\*

The authors are grateful to Lucien PAGEAU for assistance in this work.

### Symbols

$A, A_{(wt)}$	= pure water permeability constants in units of g mole water/cm <sup>2</sup> sec atm and grams water/cm <sup>2</sup> sec atm, respectively
$B$	= proportionality constant
$C$	= proportionality constant
$d$	= density of solution, grams/cm <sup>3</sup>
$d. d.$	= degree of dissociation
$(D_{AM}/K\delta)$	= solute transport parameter (treated as a single quantity), cm/sec
$f$	= solute separation defined by equation 1 or 2 as indicated
$h^{-1}$	= membrane area per unit volume of fluid space in the reverse osmosis unit, cm <sup>-1</sup>
$k$	= mass transfer coefficient on high pressure side of membrane, cm/sec
$M_A$	= molecular weight of solute
$M_B$	= molecular weight of water
$M_C$	= molecular weight of solute with respect to carbon only
$N_B, N_{B(wt)}$	= solvent water flux through membrane in units of g mole water/cm <sup>2</sup> sec and grams water/cm <sup>2</sup> sec, respectively
$P$	= operating pressure, atm or psig as indicated
[PR]	= product rate, grams/hour per given membrane area
[PWP]	= pure water permeation rate, grams/hour per given membrane area
$q_{av}$	= average product rate in weight units per unit area per unit time
$S$	= membrane area, cm <sup>2</sup> or ft <sup>2</sup>
$t$	= time in sec, or day
$\bar{u}$	= average fluid velocity parallel to membrane surface on the high pressure side of the membrane at any position in flow process, cm/sec
$\bar{u}^0$	= value of $\bar{u}$ at membrane entrance in flow process, cm/sec
$v_w^*$	= pure water permeation velocity through the membrane, cm/sec
$V_1$	= volume of solution on the high pressure side of membrane at any time in a batch process, cm <sup>3</sup>
$V_1^0$	= value of $V_1$ at the start of batch operation, cm <sup>3</sup>
$(V)_i, (V)_f$	= initial and final volumes of solutions, respectively, cm <sup>3</sup> or gallons
$x$	= longitudinal distance along the length of the membrane from channel entrance in a flow process, cm
$X$	= quantity defined by equation 29
$X_A$	= mole fraction of solute in solution
$X_{A1}^0$	= initial value of $X_A$ in feed
$X_C$	= carbon weight fraction in solution
<i>Greek letters</i>	
$\alpha_v$	= volume ratio $(V)_i/(V)_f$
$\gamma$	= quantity defined by equation 21
$\Delta$	= quantity defined by equations 26 and 27
$\Theta$	= quantity defined by equation 22

$\lambda$	= quantity defined by equation 23
$\pi$	= osmotic pressure, psi or atm
$\pi(X_A)$	= osmotic pressure of solution corresponding to mole fraction $X_A$ of solute, atm
$\pi(X_C)$	= osmotic pressure of solution corresponding to carbon weight fraction $X_C$ of solution
$\rho_1$	= density of feed solution, grams/cm <sup>3</sup>
$\rho^*$	= polar functional constant
$\sigma^*, \Sigma\sigma^*$	= Taft number
$\tau$	= quantity defined by equation 28

#### Subscripts

1, 2, 3	= refer to bulk solution and concentrated boundary solution on the high pressure side of membrane, and the membrane permeated product solution on the low pressure side of membrane at any point in the system.
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## ON-BOARD RADURIZATION OF ICED OCEAN FISH. PREPARATIONS FOR COMMERCIALIZATION IN THE GERMAN FEDERAL REPUBLIC\*

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Long distances from German ports to fishing grounds in the north-west Atlantic have created a difficult situation in the marketing of iced ocean fish. In order to arrive with fish of sufficiently good quality, the ships must return to the ports within 15 days of the first catch. This means that they must often return with incompletely filled holds. Poor utilization of the ships' capacity, together with an unfavourable ratio of fishing days to unproductive travelling days, have increasingly caused economic losses for the industry and high prizes for the consumer. The alternative of only offering frozen fish is not entirely satisfactory as most consumers still prefer iced fish.

On-board irradiation with a dose of 50–150 krad (gamma or X-rays) within 24 h of catch would at least double the shelf-life of iced fish, leading to better utilization of the ships and to better quality of the product that reaches the consumer. Preliminary trials are being carried out with an X-ray source installed aboard the research vessel "Walther Herwig". On the basis of feeding studies carried out on radurized fish in the U. K. and in Canada, on radappertized fish in the USA and on radurized marinades in Germany, and supported by microbiological studies mostly undertaken in the U. K. and the USA, a petition has been presented to the Health Ministry for clearance of ocean fish radurized on-board.

Radurization is a process designed to extend the shelf-life of foodstuffs by reducing the population of microorganisms by irradiation. Although the microbial count is reduced as a result of the radiation treatment, the product is not sterile and must be kept at refrigeration temperature. Thus radurization is always a combination treatment — radiation plus refrigeration — and may well be discussed in the context of this Symposium. The present contribution will be concerned with the application of this process to freshly caught ocean fish.

### **The need for shelf-life extension of iced fish**

The practical conditions of ocean fishing are rather different in different parts of the world. In some countries only coastal fishing is practised and fishing boats return to their ports within a few days. In other countries

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trawlers for fishing in distant waters are available, equipped with facilities for freezing. In neither case is on-board irradiation of interest. In the Federal Republic of Germany, about one-half of the sales of ocean fish reaches the market unfrozen (Table 1). Most consumers still prefer iced fish over frozen fish and the retail prices for iced fish continue to rise, while prices for frozen fish have come down. A large portion of the unfrozen fish comes from distant regions in the north-western Atlantic (Fig. 1). The return trip from these regions to German ports may require 5 or more days. Because of the limited shelf-life of iced fish, the trawlers must return within about 15 days of the first catch in order to arrive with fish of sufficiently good quality. As this leaves less than 10 days for fishing, the trawlers often return with incompletely filled holds. Rising costs, poor utilization of the ships' capacity and an unfavourable ratio of fishing days to unproductive travelling days have abolished profits of the iced-fish industry — in spite of government subsidies and in spite of increasing retail prices. If a method for extending the shelf-life of iced fish cannot be found, the trend to frozen fish (Table 1) will continue, and within a few years fresh ocean fish will no longer be available for consumers in the country's interior.

Table 1

*Sales of iced and of frozen fish in the Federal Republic of Germany  
(weight as caught)*

	1966	1967	1968	1969	1970
Total sales of iced fresh fish (1 000 t)	289	287	278	255	257
Total sales of deep-frozen fish (1 000 t)	185	188	207	221	236

### The effectiveness of irradiation

Experiments carried out in laboratories all over the world have shown that an effective method for prolonging the shelf-life of iced fish is actually at our disposal: irradiation. Due to the distance from the sea-coast, experiments in our institute at Karlsruhe have been limited to the radurization of fresh-water species (EHLERMANN & MÜNZNER, 1970) and of fish-balls obtained from frozen cod (EHLERMANN & MÜNZNER, 1969). The results have confirmed that shelf-life of iced fish can be more than doubled if a radiation dose of about 100 krad is applied while the microbial count is still low, i.e., soon after catch. Organoleptic properties are not affected by this low dose. Investigations carried out at the Federal Research Institute for Fisheries at Hamburg have led to the same conclusions (MEYER, 1971). While irradiation with considerably higher radiation doses destroys the normal spoilage flora rather completely,

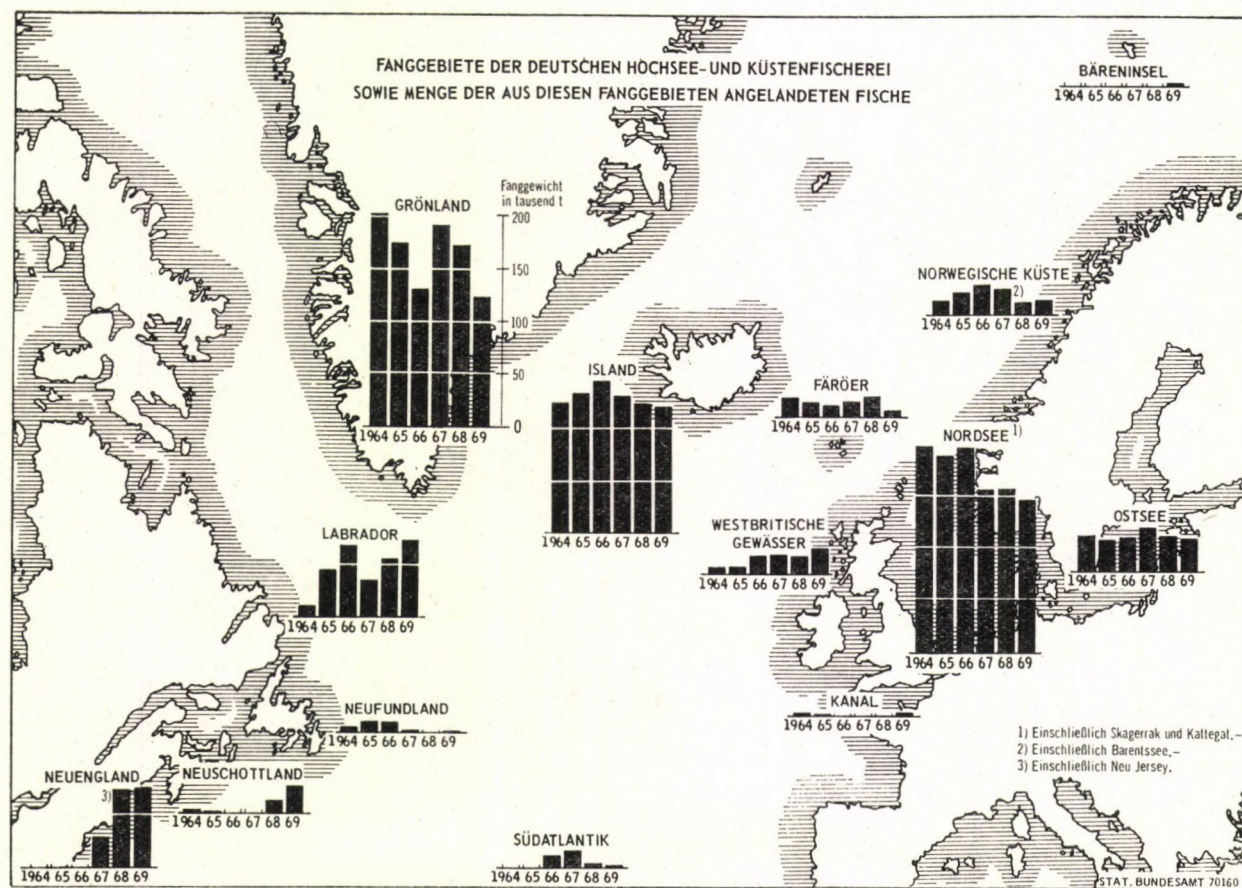


Fig. 1. Fishing areas of the German sea and coastal fisheries and the quantities landed from these areas (Annual Report on German Fisheries, 1970)



leading to atypical odours when spoilage finally does occur, fish irradiated with a dose of 100 krad produces essentially the same spoilage odour as unirradiated fish, although with much delay.

### Technological feasibility of on-board radurization

In order to irradiate fish within 24 h after catch, on-board irradiators are needed. When a pilot source was to be installed aboard the research vessel "Walther Herwig", a consideration of the advantages and disadvantages of various irradiators led to the choice of a high-power X-ray tube produced by AEG. The principles of the design are shown in Fig. 2, while a photograph of the installed facility is presented in Fig. 3. The fish to be irradiated are packed into cylindrical containers (800 mm long, with a diameter of 200 mm) and loaded from one side into a revolving magazine, which then traverses the tubular flow-through anode in the axial direction. The machine aboard the "Walther Herwig" operates at 200 kV/150 mA and has an irradiation capacity of 8 000 kg/krad/h. This corresponds to a Cobalt-60-source of about 8 000 Ci. Depending on how much the containers are filled, 50 to 80 kg of fish can be irradiated per hour with an absorbed dose of 100 krad. In contrast to an isotope irradiator, the dose rate and the hardness of the radiation can be varied by adjusting the tube current and the tube voltage, respectively. Due to the lead shielding forming part of the facility itself, the weight of the installation is only 6 tons. It was installed within a few days and without structural alterations. An isotope facility of the same irradiation capacity would have weighed more than twice as much — an unacceptable additional load for this vessel. A further advantage of X-ray facilities for use aboard

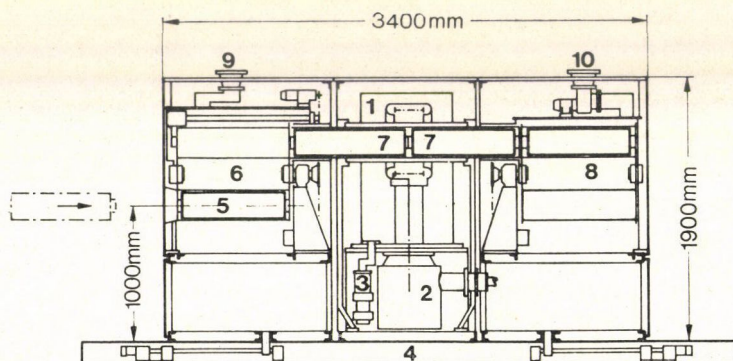


Fig. 2. Flow-through anode X-ray tube for the German fishing research ship "Walther Herwig" (Courtesy AEG). 1—Tube; 2—Transformer for cathode heating with high-voltage cable and plug 200 kV; 3—Ion getter pump; 4—Box for tube and cable conduits; 5—Irradiation container; 6—Infeed magazine; 7—Irradiation zone; 8—Outfeed magazine; 9—Air inlet; 10—Air outlet



ship is the possibility of switching off for cleaning and maintenance and in case of accidents, such as ship collisions. This is an important factor in considerations of licensing and insurance.

For commercial-scale irradiation, AEG is developing tandem X-ray facilities of the type shown in Fig. 4, in which the material is irradiated simultaneously from above and below. The distance between the two elongated X-ray tubes is variable and can be adjusted to suit the material being irradiated. Facilities of this kind are designed for operation at 200 kV and 2.5 A, with an irradiation capacity of about 300 Mrad kg/h, permitting radurization of up to 3 tons of fish per hour at 100 krad. This would correspond to a cobalt-60-source of 300 000 Ci.

Cost is an important factor in considering commercial feasibility. As no commercial-scale on-board irradiators are as yet in operation, cost calculations are based on a number of unproven assumptions. An example of the results of such calculations is shown in Fig. 5, taken from a publication by HOFMANN and co-workers (1968). Assuming a utilization time (net fishing time) of 4 000 hours per year, costs calculated for X-ray treatment amount to about 0.07 DM per kg of fish. Average auction prices for iced fish presently range from 0.50 to 0.80 DM/kg, and retail prices from 3 to 6 DM/kg. In view of the expected benefits of irradiation (better utilization of trawlers, better

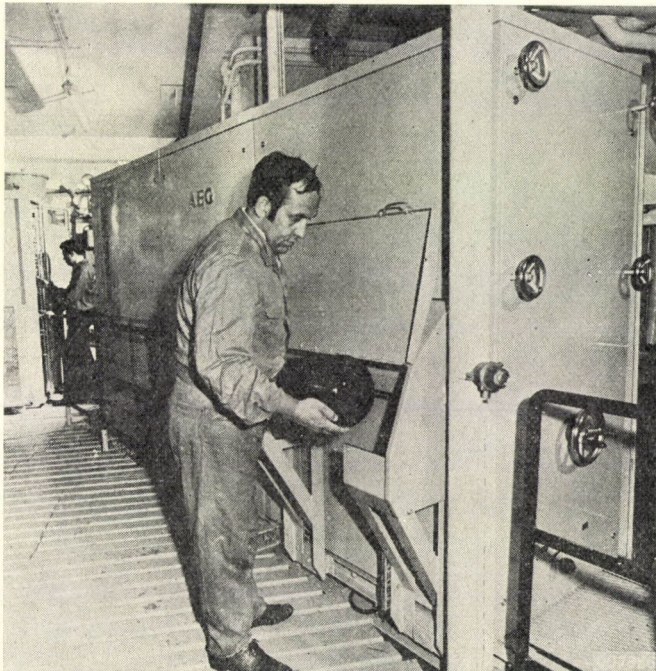


Fig. 3. Unloading of irradiation container from the high power X-ray facility aboard the "Walther Herwig". The control console is visible in the background



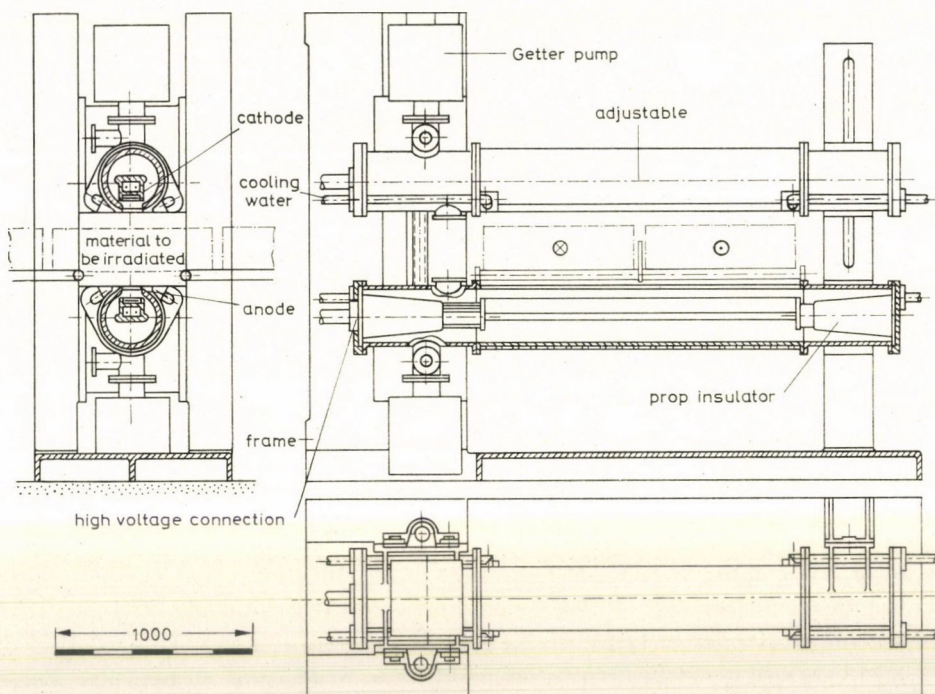


Fig. 4. Design of a tandem X-ray tube for 200 kV (HOFMANN *et al.*, 1968)

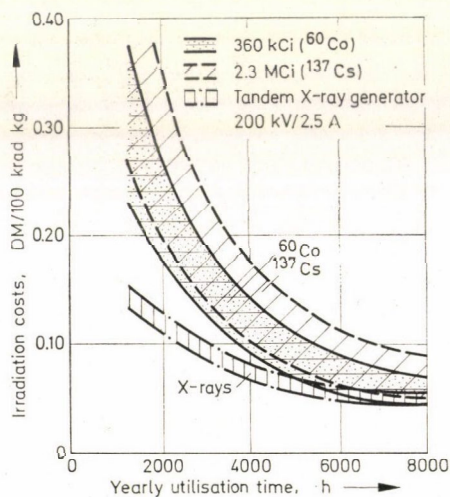


Fig. 5. Costs for radurization of fish for different radiation sources of comparable irradiation capacity. Assumed period of depreciation: 5 years (HOFMANN *et al.*, 1968)

quality of fish at auction, better supply of inland markets) the cost of irradiation appears small. These commercial aspects of radurization of fish have been presented in more detail by EHLERMANN (1973).

### **The legal situation**

As in most countries, irradiation of foodstuffs is illegal in Germany. However, the law provides for exemptions. In 1959, general clearance has been given for UV-irradiated vegetables, fruits and hard cheese. The use of ionizing radiations has been generally cleared, up to an absorbed dose of 10 rad, for process control and similar applications. Limited exemptions have recently been granted for consumer tests with potatoes irradiated for sprout inhibition and for feeding radiation-sterilized diets to intensive care patients in a hospital.

On 28 December 1971, the "Förderkreis Lebensmittelbestrahlung", an association of industrial supporters of food irradiation, has submitted a petition to the Federal Ministry of Health for permission to radurize ocean fish. The declared purpose of the radurization treatment is an extension of shelf-life on ice by at least 15 days. About half of this time is to be used for the extension of fishing trips and thus for better utilization of the trawlers. The remaining half constitutes a quality reserve, assuring a product of better quality for the consumer. The petition foresees irradiation of ocean fish (any species) within 24 hours of catch, with a dose of 50 to 150 krad. It consists of a bound volume of over 100 pages; the claims for the feasibility of the process and for the wholesomeness of irradiated fish are documented by over 200 literature references. The Ministry has asked numerous experts to evaluate this petition and to give their advice; a decision is not expected before 1973.

### **The problem of wholesomeness**

Decisions of this kind are based on two principal considerations: wholesomeness and technological need. The latter cannot be disputed under the conditions prevailing in the German fisheries. The former is much more difficult to establish. The petition by the "Förderkreis" quotes numerous studies on the chemical composition of irradiated fish, all indicating that the changes caused by irradiation, even in the Megarad-range, are quantitatively so small that they can be detected only by very sensitive methods. However, even the most thorough chemical analysis cannot exclude the presence of some unthought-of toxic compound. Animal feeding studies therefore remain to be the backbone of toxicological considerations. Judgements on the whole-



someness of irradiated fish can be based on very extensive feeding studies (Table 2).

Table 2  
*Animal feeding studies with irradiated fish*

Species of fish	Dose (Mrad)	Species of test animal		
		Rat	Dog	Mouse
Cod	0.6	Hickman, 1965 Hickman, 1966 Hickman <i>et al.</i> , 1969a Hickman <i>et al.</i> , 1969b Hickman <i>et al.</i> , 1969c	—	Hickman <i>et al.</i> , 1969b
	2.8 and 5.6	Newberne, 1959, Alexander and Salmon, 1959	Alexander and Salmon, 1959, Newberne and Salmon, 1960	Calandra and Kay, 1963
Haddock	0.2	—	—	Procter <i>et al.</i> , 1971
	2.8 and 5.6	Read <i>et al.</i> , 1961	—	—
Tuna	2.8 and 5.6	Ross and Hood, 1963, Paynter, 1959	McCay and Ramsay, 1960, Ross <i>et al.</i> , 1962	Deichmann, 1963
Herring (marinated)	0.16 and 0.48	Schönborn <i>et al.</i> , 1970	—	—

None of these studies have given indications of toxic or carcinogenic effects of irradiated fish. The investigation with herring marinades, which was carried out at the Battelle Institute, Frankfurt, while not showing abnormalities in the test animals as judged by the usual criteria of health (growth, fertility, histology, etc.), did indicate an effect of the irradiated diet on cortical chronaxia, *i.e.*, on central nervous excitability; no effect on peripheral excitability was found. The significance of this observation is uncertain. Experiments designed to clarify this point are being carried out at Battelle. One possible explanation is, in my opinion, a marginal vitamin B<sub>1</sub>-deficiency in the test-animals.

A recent report by SHILLINGER and OSIPOVA (1970), who claimed to have found unfavourable effects of an irradiated fish diet on rats and who had concluded that fish irradiated at a dose of 600 krad cannot be recommended for human consumption can hardly negate the results of the numerous investigations quoted in Table 2. These authors compared unirradiated fish, which was kept frozen, with irradiated fish (600 krad), which was kept for up to 2 months at temperatures of  $2 \pm 5^\circ\text{C}$ . In this temperature range a serious loss of quality must be expected when fish (even if irradiated) is



stored for two months. The presumed effects of the irradiated diet on enzyme activities in blood and liver may rather have been effects of a spoiled diet. Moreover, from a statistical viewpoint, the data presented in the publication are quite insufficient to support the claimed effects. The authors' remark that their rats were infested by parasites (helminths) sheds additional doubt on the validity of their conclusions.

An important aspect of the wholesomeness of radurized fish is microbiological safety. Theoretically, radiation may destroy the harmless spoilage flora, which normally competes with the development of *Clostridium botulinum*, leaving only the highly radiation resistant *Clostridium* spores. This possibility is not unique to irradiation. Spores are also very resistant to heating and smoking and these treatments may also favour *Clostridium* by destruction of competitors. Nevertheless, this situation has been thoroughly studied in irradiated fish. It can now be stated with confidence that the conditions foreseen for on-board irradiation will not increase the risk of botulism for the following reasons:

1. The fish is to be kept in ice at all times. It is well known that at this temperature (not over 3 °C, even in the outer layer of containers), no *Clostridium* toxin can be formed.

2. Substantial counts of *Clostridium botulinum* are only found in waters close to coastal areas. Fish caught on the high seas is essentially free of the various *Cl. botulinum* types.

3. Even if these conditions did not prevail, irradiation with a dose of about 100 krad would not substantially increase the risk, as this dose is low enough to leave a mixed microbial population which can compete with the growth of spore forming bacteria.

Summarizing the results of extensive microbiological studies which were mostly carried out in the U. K. and the USA, experts convened at an IAEA Panel Meeting have concluded: "Radurization of fresh fishery products is a feasible process if the final products are stored below 3 °C, achieving a two or three times increase in shelf-life with no known health hazard." (ANON, 1970).

### Prospects for commercialization

If present trials with the pilot-scale on-board irradiator continue to produce favourable results, the next step should be the installation of a high-capacity irradiator aboard a large trawler, followed by commercial-scale shipping studies and test marketing. This requires general clearance of fish irradiation according to the petition of the "Förderkreis" — or at least an



exemption which permits limited marketing of irradiated fish in substantial quantities. At any rate, a favourable judgement on the wholesomeness of irradiated fish by the Health Ministry will be the precondition for all further developments.

For a number of years, the wholesomeness problem has been recognized as the central obstacle to any progress in the field of food irradiation. This recognition has led to the creation of the International Project, sponsored by OECD and IAEA and jointly financed by 21 nations. Under the able leadership of R. Hickman, a toxicologist on leave from the Canadian Food and Drug Directorate, the Project is presently devoting its resources to several feeding studies with irradiated potatoes, wheat and fish. Other products are to be tested in coming years. If this Project does not succeed in demonstrating the safety of irradiated foods to the satisfaction of national and international health authorities, all further efforts in this field will be doomed. The task is a formidable one: how is it possible to prove beyond any doubt that a food treated in a particular way is safe to eat? Recent developments, such as experiments casting doubts on the safety of cyclamates, have strengthened the attitude that there is no "absolute safety". A margin of risk remains, even when the most sophisticated experiments have not given evidence of toxicity. How much risk is acceptable in view of expected benefits? It is impossible to predict how a person or a committee or a ministry will answer this question in a particular case. It seems only safe to say that the present atmosphere of fear of real or imagined environmental dangers is not conducive to innovation in food technology. It has become increasingly difficult in recent years to get approval for new processes or new additives. Once the health authorities have been convinced that fish irradiated with a dose of 100 krad is wholesome, it should be possible to convince the consumer — by nothing but the superior quality of irradiated fish.

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## INFLUENCE OF RELATIVE HUMIDITY CONDITIONS ON THE THERMAL RESISTANCE OF SEVERAL KINDS OF SPORES OF MOLDS\*

M. LUBIENIECKI-VON SCHELHORN

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The thermal resistance of conidiospores of *Aspergillus niger*, chlamydo-spores of *Humicola fuscoatra*, and ascospores of *Byssoschlamys fulva* in water and under atmospheres of relative humidity from 0 to 60% has been studied. From the experimental results *D* values referring to 100 °C were calculated for several levels of relative humidity. On this basis a comparison was made between the mentioned resistant bodies of molds against each other and against heat resistant bacterial spores.

The thermal resistance of the most resistant spores of molds is decidedly lower than that of the most resistant spores of bacteria: Under equal conditions of temperature and relative humidity the *D* values belonging to the mold spores are about ten times lower than those belonging to the bacterial spores.

Similar to spores of bacteria the heat resistance of mold spores proved to be much higher when thermal treatment was carried out in dry than in aqueous substrates.

Contrary to bacterial spores which, according to several authors, possess highest thermal resistance between 20 and 40% relative humidity, mold spores show continually decreasing heat resistance with increasing relative humidity over the whole range from 0 to 100%.

The conidiospores of *Aspergillus niger* show much lower resistance at every level of relative humidity than the resistant bodies of *Humicola fuscoatra* and *Byssoschlamys fulva*. Compared against each other the latter two species showed similar heat resistance.

In recent years manifold research has been done on the heat resistance of bacterial spores as dependent on relative humidity. The main results of these investigations are:

The period of time required to reduce the number of spores by 90% (*D* value) is by far longer with heat treatment in an atmosphere of lower humidity than in aqueous liquids or in water vapour saturated atmosphere. More accurately, according to MURRELL and SCOTT (1966) and ANGELOTTI and co-workers (1968), the heat resistance is highest with 20 to 40% of relative humidity, not with 0%. The work done in our laboratory deals with the behaviour of resistant bodies of molds under thermal treatment in atmospheres of different relative humidity and, to be compared with, in water.

\* Presented at the IUFoST Symposium on Combination Treatments in Food Preservation, Budapest 18—22 September 1972.



The following resistant bodies of molds were used in our experiments: conidiospores of *Aspergillus niger* (from our own collection); chlamydospores of *Humicola fuscoatra* (from an industrial laboratory); ascospores of *Byssoschlamys fulva* (from Dr. Senser, Munich).

It is known, that the latter two types of spores extremely resist heat treatment in the presence of water or water vapour. It seemed, therefore, of interest to learn about their behaviour in arid environment.

Apart from the scientific point of view, we were interested in the question at what temperature spores of molds clinging to packaging materials could be destroyed. If those materials could be freed of mold spores by dry thermal treatment it would be possible to use them for aseptic packaging of acid beverages, even if spores of heat resistant bacteria should remain surviving on them.

### 1. Materials and methods

The technique used throughout our work for counting the spores before and after heat treatment was the usual plate culture procedure. Some of the species gave rise to difficulties here. This led to particular modifications of the procedure.

The conidiospores of *Aspergillus niger* were grown on malt agar, knocked off as a dust, mixed with quartz sand and thus weighed for each variant of experiment. The conidiospores of *Aspergillus niger*, in general, remained single, as confirmed by microscope. Their destruction by heat seems to follow more or less the exponential law. On that basis it was possible to derive thermal death time curves from a series of counts made at different times of the heating process by means of the standard least squares procedure. From these curves the *D* values (periods of time required for reduction by 90%) were calculated.

*Humicola fuscoatra* was grown on malt agar too. Here the surface layer containing the mycelium was scratched off together with the chlamydospores, wet-ground with sea sand, followed by filtering through cheese cloth. And yet, as the microscope revealed, not all of the chlamydospores in the resulting filtrate were single. Thus, transformation into plate cultures would not furnish countings directly representative of the true numbers of the chlamydospores per volume unit of filtrate. Nevertheless, an indirect approach of the true number of chlamydospores per volume unit of filtrate was found possible from plate-countings in the following way:

By aid of microscope the relative numbers of single spores, of groups of two, of three, and so on, were determined. These were accounted for as follows:

$$\begin{array}{l} \text{True number of} \\ \text{chlamydospores} \\ \text{per volume unit} \\ \text{of filtrate} \end{array} = \begin{array}{l} \text{percentage of single spores} \\ + 2 \text{ (percentage of twins)} \\ + 3 \text{ (percentage of triades)} \\ \text{count of spots} \\ \text{on agar} \end{array} + \frac{\dots\dots\dots}{100}$$

For the "dry" series, requiring water-free chlamydospores, the filtrate was distributed in amounts of 0.1 ml into small glass tubes — the details of which are given below — and subjected to freeze-drying. No certainty could yet be obtained as to how easily re-clustering may happen by freeze-drying and by heat-treating the dried samples. Furthermore, in the case of chlamydospores, which are relatively large, the process of death may be of a multistage character. This would render the thermal death time curve non-exponential. For these reasons it was given up to construct thermal death time curves. Instead, thermal treatment was continued until only one mold spot was obtained from every 2 750 spores (as a mean) contained in 0.1 ml. In this case it is highly probable that this spot originates from a single spore, left over alone from a group. The original equation

$$D = \frac{t}{\log N_0 - \log N_t} \text{ then may be transformed into } D = \frac{t}{\log N_0}.$$

In case of non exponential destruction the  $D$  value thus obtained is no longer valid for a part of the thermal death time curve, but still holds as a mean for the whole. MOATS and co-workers (1971) recommend, wherever the thermal death time curves should not appear truly logarithmic, to establish " $F$  values", that is "the time required for a given probability of killing at a given temperature". The " $D$  value" referred to in our own report is equal  $\frac{F}{\log N_0}$ .

We chose to transform the " $F$  values" to values of "average  $D$ " to enable us to compare the values concerning *Humicola fuscoatra* with our own results concerning the other two species of molds and with the results of other authors.

The ascospores of *Byssoschlamys fulva* were handled similarly to *Humicola fuscoatra*. In the filtrate prepared from the mother-culture, single spores were found as well as intact asci. The asci are known to contain 8 spores each. Again the numbers of free spores (ascospores) and groups (of eight) were assessed by the help of the microscope and taken into account as follows:

$$\begin{array}{l} \text{True number of} \\ \text{ascospores} \end{array} = \begin{array}{l} \text{percentage of singulars} \\ + 8 \text{ (percentage of asci)} \\ \text{count of spots} \end{array} \cdot \frac{\dots\dots\dots}{100}$$



The heat treatment was carried out in the following way: For moist heat treatment the suspension of spores in water was poured into glass tubes of 65 mm length, 7 mm inner diameter and 0.7 wall thickness, wherein they were heated. Glass tubes of equal design also served for dry heat treatment of spores. These open tubes containing spores in water or dry spores were immersed to the brim into the bath of a thermostat. Heating dry spores in open tubes has the advantage of being more simple than drying over phosphorus pentoxide, as described later, but it implies the disadvantage that the relative humidity in the vicinity of the spores can hardly be adjusted with exactness. If dry spores are heated in open tubes, the air next to the spores attains a relative humidity which depends on the relative humidity of the atmosphere, on its temperature and on the temperature to be arrived at for treatment. In our experiments the relative humidity of the air surrounding the spores lay somewhere below 2% relative humidity as a result of the procedure. With this design of experiment used, the treatment temperature was reached within maximum 2 minutes. To keep this interval negligible heat treatments were not shorter than 20 minutes.

This simpler method of dry heating in open tubes was utilized for obtaining as many *D* values as possible in order to calculate *z* values as explained afterwards.

A better adjustment of relative humidity in the environment of the spores regardless of temperature is secured by a method published by MURRELL and SCOTT (1966) which was adopted. Fig. 1 shows the device used.

Of the two tubes the smaller one houses the spores, the larger one is charged with phosphorus pentoxide (granting 0% of relative humidity) or with sulfuric acid of different concentrations. Over sulfuric acid distinct relative humidities are established depending on the concentration of the acid and independent from temperature. The outer glass jacket was sealed after evacuation. Thus the spores were stored for several weeks to attain equilibrium

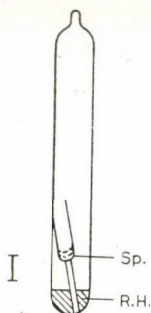


Fig. 1. Apparatus for determining heat resistance of spores at various relative humidities Sp. = Spores; R. H. = Medium for establishing relative humidity inside the tube ( $P_2O_5$  or  $H_2SO_4$ ). The bar I is equivalent to 1 cm

with respect to relative humidity. After that heat treatment was applied. These double tubes were submerged entirely in the bath of a thermostat. With this arrangement the treatment temperature could be reached within 10 minutes. Here heat treatment periods not shorter than 2 hours were employed to keep the heating-up interval negligible. Accordingly, the temperature of treatment had to be chosen in such a way that periods from 2 to 8 hours were going on, to result in evaluable effects of spore number reduction.

The thermal treatment was followed by quick chilling in an ice-water mixture, whereupon the preparation of the plate-cultures was performed.

$z$  values (= the increase in temperature which decreases the  $D$  value by a factor of ten) were calculated from  $D$  values, each one of which was obtained at three temperatures of dry or moist heating, by means of the formula

$$z = \frac{T_2 - T_1}{\log D_1 - \log D_2}.$$

## 2. Results

Table 1 shows the  $D$  values (minutes) obtained from the thermal treatment of conidiospores of *Aspergillus niger* and chlamydospores of *Humicola fuscoatra* at different temperatures and relative humidities.

The table does not include any direct information on statistical probability. This is due to the impossibility of repeating the experiments often enough to secure definite numerical values of statistical probabilities of the

Table 1

*D values (minutes) of conidiospores of Aspergillus niger and chlamydospores of Humicola fuscoatra at different temperatures and relative humidities*

### 1a) *Aspergillus niger*:

Rel. humidity %	0	2.5	5	10	20	30	60	100
Temperature °C	100	100	90	90	80	80	70	55
$D$ value min.	100	85	165	105	210	216	100	6

### 1b) *Humicola fuscoatra*:

Rel. humidity %	0	2.5	10	30	60	100
Temperature °C	120	120	110	100	80	80
$D$ value min.	30	28	47	100	143	101

Results obtained by treatment in double-tubes, as described above and shown in Fig. 1



various results. This did not seem necessary, only the comparison of the heat resistance values at different relative humidities being of interest and not the absolute "*D*" values. The latter do not only depend on temperature and relative humidity, but also on other factors, as *e.g.* strain characteristics or on the age of the spores. As expected, absolute values frequently showed noticeable variations while the ratio of the *D* values and similarly the *z* values calculated therefrom, remained constant over a wide range of relative humidities.

The ascospores of *Byssoschlamys fulva* gave a *D* value of 25 min at 0% relative humidity and 120 °C. Experiments with higher relative humidities were not carried out with *Byssoschlamys fulva*. Table 2 shows *z* values for dry and moist thermal treatment of conidiospores of *Aspergillus niger* and chlamydospores of *Humicola fuscoatra* and for dry thermal treatment of ascospores of *Byssoschlamys fulva*.

Table 2

*Some z values (°C) for dry and moist thermal treatment of conidiospores of Aspergillus niger and chlamydospores of Humicola fuscoatra and for dry thermal treatment of ascospores of Byssoschlamys fulva*

Species	<i>z</i> value °C (dry heating)	$\frac{t \cdot s}{\sqrt{n}}$ (95% prob.)	<i>z</i> value °C (moist heating)	$\frac{t \cdot s}{\sqrt{n}}$ (95% prob.)
<i>Aspergillus niger</i> (conidiospores)	12	5	3.9	0.7
<i>Humicola fuscoatra</i> (chlamydospores)	28	12	10	5
<i>Byssoschlamys fulva</i> (ascospores)	17	8		

Definition of *z* value: The increase in temperature necessary to decrease *D* values (valid for a given species and relative humidity) by a factor of ten

Every *z* value of Table 2 is the arithmetic mean of two "raw" *z* values pertaining to a pair *D*<sub>1</sub> and *D*<sub>2</sub> and to a pair *D*<sub>1</sub> and *D*<sub>3</sub> of *D* values which had been found at the temperatures *T*<sub>1</sub>, *T*<sub>2</sub> and *T*<sub>3</sub>. Therefore, the limits of statistical precision must be rather wide, apart even from these "refined" *z* values, each one of which being the average of only two data. But more *D* values, belonging to further temperatures, were not available because of the limitations of the experiment.

### 3. Conclusions

It can be seen from Table 1 that treatment temperatures resulting in manageable treatment periods are much higher in the case of *Humicola fuscoatra* than of *Aspergillus niger* at any value of relative humidity. This evidently is due to the fact that the chlamydospores of *Humicola fuscoatra* are by far the more heat resistant ones. As to relative humidity, the higher the latter, the lower temperatures had to be chosen for the determination of the  $D$  values of both of the species. This was because heat resistance decreases with increased relative humidity. A synopsis of all  $D$  values referring to one temperature can be arrived at only by calculation. The key to it is the obtention of the  $z$  value.

It seems fairly safe, furthermore, to deduce from Table 2 that  $z$  values obtained on the basis of dry heating range distinctly above those obtained with moist heating. This fits in well with various findings of other authors, e.g. of MURRELL and SCOTT (1966) who made comparable observations with bacterial spores. Nevertheless, the determination of the  $z$  values with any better than "rough" precision involves very substantial difficulties as is generally true, not only in our own research work. As found in literature, e.g. one of the authors reports a  $z$  value of 33 referring to dry thermal treatment of *Bacillus subtilis*, whereas another author determined the same value as 17. However, we had to use these rough  $z$  values, if we wished to calculate  $D$  values at uniform temperature.

100 °C  $D$  values related to several levels of relative humidity were calculated by the aid of Tables 1 and 2 for *Aspergillus niger* and *Humicola fuscoatra* and therefrom the curves of Fig. 2 were drawn. In the median range of humidities  $z$  values could only been extrapolated from those related to extremely moist and extremely dry treatments. It is obvious, for reasons explained before, that Fig. 2 is not intended to give detailed information. Still, following further definite statements can be read from the figure.

The figure confirms what had been said in discussing Table 2, namely that the chlamydospores of *Humicola fuscoatra*, at any relative humidity, are more resistant than the conidiospores of *Aspergillus niger*. The difference with respect to the  $D$  value at 100 °C between dry and moist heat treatment is greater with *Aspergillus niger* than with *Humicola fuscoatra*. The tested spores of *Aspergillus niger* and *Humicola fuscoatra* showed decreasing resistance with increasing relative humidity. A particular range of the scale of humidities giving highest resistance, as reported for bacterial spores, does not seem to exist with molds.

Also for the ascospores of *Byssoschlamys fulva* the  $D$  values pertinent to 0% relative humidity and 100 °C were calculated. While at 120 °C the resistance of *Humicola fuscoatra* seems to be somewhat stronger than that



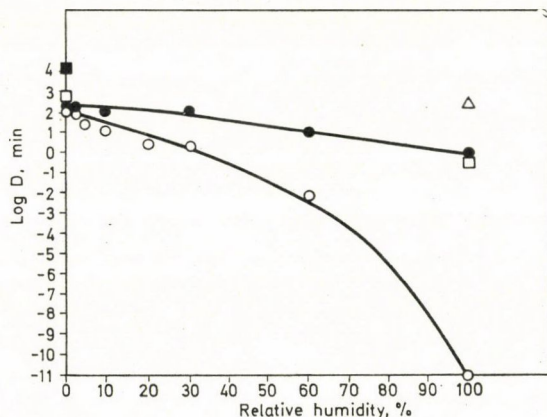


Fig. 2. Comparison of the approximate  $D$  values of persistent forms of molds and bacteria at 100 °C as dependent on relative humidity. The values of *Aspergillus niger* (at any relative humidity), *Humicola fuscoatra* (at any relative humidity), and *Byssoschlamys fulva* (dry heating) are calculated from results of experiments of the author (Tables 1 & 2 in the text); the value for *Byssoschlamys fulva* (moist heating) is calculated from data published by KING and co-workers (1969); the value  $D$  of *Bacillus subtilis* (dry heating) is calculated from data published by FOX and EDER (1969); the value of *Bacillus stearothermophilus* is calculated from information given by MURRELL and SCOTT (1966). ○ *Aspergillus niger*; ● *Humicola fuscoatra*; □ *Byssoschlamys fulva*; ■ *Bacillus subtilis*; △ *Bacillus stearothermophilus*

of *Byssoschlamys fulva*, at 100 °C *Byssoschlamys fulva* seems to be more resistant. But this may be traced to the fact that not the same  $z$  value was applied in the case of the two species. In Fig. 2 a  $D$  value related to moist heat treatment of *Byssoschlamys fulva* is also recorded which had been converted to the 100 °C value by utilizing information published by KING and co-workers (1969). Like with *Humicola fuscoatra* also with *Byssoschlamys fulva* the difference of  $D$  values of moist and dry heat treatment at 100 °C seems to be smaller than with *Aspergillus niger*.

Finally, another safe statement derived from our experiments is that the chlamydospores of *Humicola fuscoatra* and the ascospores of *Byssoschlamys fulva* possess considerable resistance against heat treatment in the dry state, but that even this resistance of exceedingly heat resistant mold spores does not reach the degree of the more resistant bacterial spores. As it is known, as far as moist environment is concerned, *Bacillus stearothermophilus* is the most resistant, while in dry atmosphere *Bacillus subtilis* (LUBIENIECKI-VON SCHELHORN, 1972).  $D$  values related to 100 °C for these bacteria, collected from literature, are recorded as dots in the figure.

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## PRESERVATION OF CANNED VIENNA SAUSAGE BY COMBINATION OF HEAT AND RADIATION\*

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Since no texture improver is permitted in Hungarian Vienna sausage, the heat treatment necessary for bacteriological safety causes texture and consistency problems with this type of product. The aim of the investigations was to lower the heat damage by using a combination of mild heat treatment and irradiation. During the experiments an irradiation dose of 0.45 Mrad was combined with heat treatments of various  $F_0$  values (0.2—0.5).  $F_0$  of the control was 1.9. Naturally contaminated samples were used. Storage temperature was 30 °C, storage time up to 2 months.

Combination of irradiation (0.45 Mrad) and heat treatment ( $F_0 = 0.4$ ) — regardless of the sequence — gave satisfactory results in shelf-life and excellent results in organoleptic properties as compared to traditionally heat treated samples. This statement is valid for uninoculated samples only.

Because of the poor heat conductivity and high initial microbial count of ingredients, the traditional preservation method (canning) causes texture and consistency problems. These difficulties can be overcome with various firming agents, if permitted. In Hungary, nevertheless, no such additive is allowed to be used in Vienna sausage, which is therefore especially sensitive to heat treatment from the points of view of texture and consistency. In order to obtain canned Vienna sausage of good quality, heat treatment should be lowered. This can be done — among others — by using a combination of mild heat treatment and irradiation. It has been found by MORGAN and REED (1954) and KEMPE *et al.* (1954) that certain combinations of heat and irradiation exhibit a synergistic effect on the death of bacteria and the surviving but injured bacterial spores become more sensitive to the inhibiting effect of curing additives than untreated ones. It seems thus possible to ensure satisfactory shelf-life and microbiological safety by combination of such mild heat and irradiation treatments which when separately applied, do not have sufficient keeping effect. Since in this combination the heat treatment is mild, the quality of the final product ought to be higher than that of the traditionally treated one. Beside the combination treatments, also tyndalization has been tested.

\* Presented at the IUFOST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



## 1. Materials and methods

Skinless Vienna sausage was put in 6 oz. ( $\sim 200$  g) cans (three in each) and was filled with a brine containing 3% NaCl, 0.1% potassium nitrate and 0.2% tartaric acid. Vienna sausage was produced with nitrite in the pilot plant of the Hungarian Meat Research Institute. The ratio of brine and sausage was approx. 1 : 1. pH of brine was between 5.4–5.5,  $a_w$  of the canned products was 0.965. Naturally contaminated and inoculated samples were used; this paper presents only data of the first group.

Heat treatment of cans was carried out in an autoclave and a  $^{60}\text{Co}$  gamma source of 45 kCi has been used for irradiation.

Treatments were as follows:

- heat treatment at an  $F_0$  value of 1.9 (110 °C 30') which served as the control;
- tyndallization: 30' at 100 °C twice, with 2 hrs rest in between.  $F_0$  value  $\sim 0.4$ ;
- 30' heating at 100 °C ( $F_0$  value  $\sim 0.2$ ) plus irradiation with a dose of 0.45 Mrad;
- same as above but sequence changed.

Each batch consisted of 50 cans, ten of which were used for organoleptic tests, and the experiment was repeated once. Since heat treatment and irradiation were not carried out at the same place, 2 hrs have elapsed between treatments.  $F_0$  values were estimated according to HEIDTMANN and REICHERT (1969).

Preliminary experiments have also been carried out in order to determine the effect of incubation time between two fractional heat treatments and of nitrite on keeping quality. In these investigations 25–25 cans with 3 sausages in each were used in a batch, and the treatments were as follows:

1. 30' at 95 °C twice with no incubation in between;
2. 30' at 95 °C twice with 2 hrs incubation in between.

These two treatments were carried out both in brine as mentioned above and in brine containing, instead of nitrate, 600 ppm nitrite and 3% NaCl. (The value of 600 ppm decreased to 300 ppm after equilibration.)  $F_0$  value was approx. 0.25. In all the experiments cans were incubated at 30 °C, blown cans were removed and counted.

In order to determine the effect of various treatments on quality, organoleptic tests were carried out. The quality was judged by a taste panel of 7 well-trained members. The warmed up Vienna sausage was tested according to the following scale:

- 5 = excellent
- 4 = good
- 3 = satisfactory
- 2 = acceptable
- 1 = non-acceptable.

The results of these tests were evaluated by analysis of variance.

In addition to organoleptic tests also objective measurements of texture were made with the aid of the "Texturometer" (Zenken Co., Japan). In these experiments Vienna sausage was prepared the same way as above, texture profiles of 12 mm slices were tested and registered.

## 2. Results

The results of combination treatment are presented in Fig. 1. It is clear, that in the 1st series of experiments no spoilage occurred after 2 months of storage. In the 2nd series some spoilage occurred, this was caused mainly by aerobic spore-formers. (It should be noted that spoilage of one single can meant 2.5% spoilage as a consequence of low sample-number.)

The results of organoleptic tests are to be seen in Fig. 2. These results were evaluated by analysis of variance, according to which the samples first heated and irradiated thereafter are of the highest quality, the difference is significant at the level of 0.1%. The samples first irradiated, then heated and the samples heated in a tyndallization process were worse than the 1st group and better than the control. Both differences were significant. No

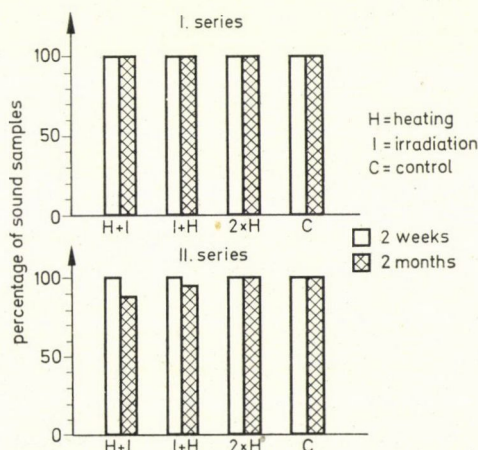


Fig. 1. Effect of various treatments on shelf-life of canned Vienna sausage. Storage period: 2 weeks and 2 months. H:  $F_0 = 0.2$ ; I: 0.45 Mrad; C:  $F_0 = 1.9$



difference could be found, however, between the tyndallized, heated and irradiated plus heated samples.

No difference has been found among panel members and neither treatments nor replicates influenced the results of organoleptic tests. The two treatments giving medium quality gave results closer to control samples in the first series and closer to irradiated plus heated samples in the second series, but difference is significant in both cases. This phenomenon appears in the significant interaction between replicates and treatments, this interaction is, nevertheless, of little importance.

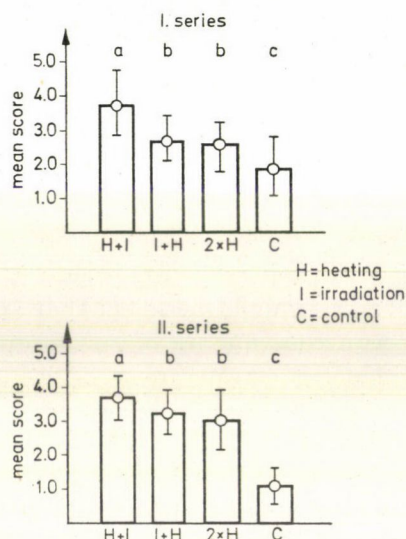


Fig. 2. Effect of various treatments (same as in Fig. 1) on sensory quality of canned Vienna sausage (differences are significant between a, b and c)

The texture differences among variously treated samples measured with the Texturometer are shown in Fig. 3.

As is the case with organoleptic tests the hardness of the control was significantly lower than that of the other samples.

Concerning our preliminary tests with the effect of nitrite on keeping quality some considerations and the results are to be mentioned.

Having supposed the equilibration between nitrite content of brine and sausage and knowing the breakdown of nitrite during heating, 600 ppm were used in the brine. (This falls to an initial level of about 300 ppm after equilibration.) It should be emphasized once more that these were preliminary tests and we started from above the highest nitrite concentration, the residue of which health authorities might tolerate at all. (Between 150–200 ppm.) Besides, low  $F_0$  has been used in order to avoid masking of nitrite-effect

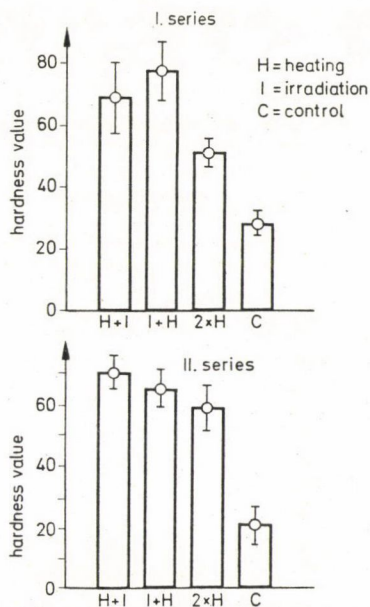


Fig. 3. Effect of various treatments on hardness values of canned Vienna sausage measured by Texturometer (treatments are same as shown in Fig. 1)

by heat treatment. This nitrite effect can be proved namely only with low  $F_0$  values, if no inoculation is used. The results are shown in Fig. 4.

In the 1st series after 5 weeks of storage 60% of the samples were sound when containing nitrite, the control was spoiled to 100%, in the 2nd series the differences can be seen, too.

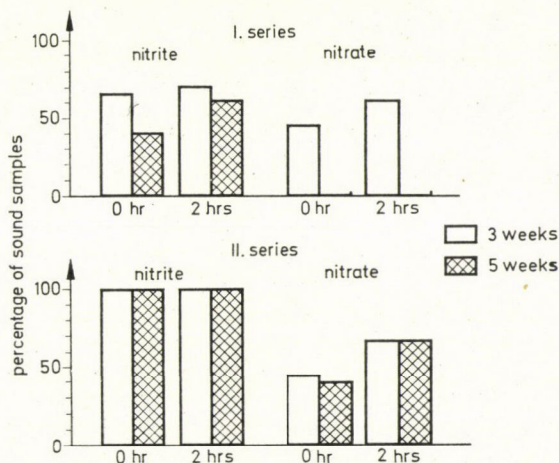


Fig. 4. Effect of curing additives on stability of canned Vienna sausage (nitrite: 300 ppm initial  $\text{NaNO}_2$ ; nitrate: 0.1% initial  $\text{KNO}_3$ ; heat treatment:  $F_0 \sim 0.25$ )



## 2. Conclusions

From the experiments it was evident that a fraction of traditional heat treatment —  $F_0 \sim 0.2$  — gives fairly satisfactory microbiological stability when heat treatment is combined with mild irradiation.

Tyndallization with low  $F_0$  value gave good shelf-life, too.

It should be kept in mind, though, that these experiments were carried out with a rather low number of samples and without artificial inoculation. In order to get more reliable data experiments were carried out with inoculated samples, too, the results of which will be presented in the next volume of *Acta Alimentaria* (FARKAS, INCZE & ZUKÁL, 1973).

The main merit of combination treatments is that they yield good organoleptic quality, highly significantly better than traditional heating. It is interesting to note, that in combination of heat and irradiation, the sequence of treatment influenced organoleptic quality as well, this being significantly better when heat treatment came first. At the same time no such difference could be found with Texturometer tests, probably, because panel members were influenced not only by the hardness of the sample. The sequence-dependent difference in organoleptic quality between combination treated samples might perhaps be explained by the higher sensitivity of less denaturated protein towards irradiation.

It is worth noting how low an  $F_0$  value yielded satisfactory keeping quality in our experiments supporting thus the results of other authors (HEIDTMANN, 1970).

Since — in spite of microbiological stability — the survival of clostridial spores with this heat treatment cannot be excluded at all, the importance of curing salt, first of all of nitrite is vital.

It is also worth mentioning that tyndallization-like heating, with as short as 2 hrs incubation between treatments makes possible a part of surviving spores to germinate, resulting in a higher destruction during the 2nd treatment and a better keeping quality as well.

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## SANITATION OF ONION POWDER BY IRRADIATION AND HEATING\*

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Onion powder is frequently contaminated by a mesophile bacterial flora containing some spore-forming strains. Heating is not an admissible method of sterilization in this case, because of the volatile aromatic substances formed by the enzyme system present.

After irradiation with 0.8 Mrad the surviving microbial flora contained *Clostridium*, *Bacillus*, *Enterococcus* and *Micrococcus* strains. These *Micrococcus* strains are less resistant than *M. radiodurans* and do not develop as spoilage organisms in foods. During the months following irradiation no growth was observed, on the contrary the number of cells surviving diminished in the same way both in the irradiated and the untreated samples.

The unwholesome microbial flora introduced with the onion powder varies with the product in which it is used.

In soup powders with short cooking time, if the irradiated or untreated onion powder is added after boiling, a change is caused by the growth of *Lactobacillus* or *Bacillus* strains. A radiation treatment with 0.4 Mrad gives a sterile soup, when boiled for 5 minutes.

In meat pastes the most dangerous microbial flora is that consisting mainly of spore-forming anaerobic bacteria. It was shown that if the paste was aromatized with onion powder treated with 0.2 Mrad before cooking, no growth occurred. The control sample containing untreated onion powder was spoiled by a microbial flora dominated by spore-forming aerobic bacteria.

These two experiments proved that onion powder can be decontaminated by treatment with 0.2 or 0.4 Mrad. It is known that this dose has no detrimental effect on the organoleptic quality of the onion powder and the cost of irradiation is compatible with the price of the product treated.

Onions are dried without prior blanching as they must retain some enzymatic activities to hydrolyse the sulfur containing products and produce by this reaction the characteristic flavour. As a result the products thus obtained are sometimes highly contaminated by microorganisms. Thus utilization of onions in the state of incipient deterioration may be considered a typical case.

When onion powder is incorporated as seasoning in foods which are not sterilized it can produce contaminations which are not compatible with the requirements of technology.

The microflora in the samples of dried onions from Mexico and California has been studied by VAUGHN (1970); coliform bacteria, pectinolytic bacteria,

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lactic acid bacteria and aerobic as well as anaerobic spore-formers have been identified. It was concluded that one or the other group of these bacteria would be troublesome according to the type of food in which the onion powder is used, for example: thermophilic spore-formers in canning, psychrophilic bacteria in refrigerated foods, pectinolytic bacteria in pickles.

Onions contain among various disulfides, propyl-propyl and propyl-allyl disulfides which are bactericidal to *E. coli* and *S. typhimurium* (JOHNSON & VAUGHN, 1969). It is not surprising that VAUGHN (1970) has never found *E. coli* in the 293 samples of dried onions and garlic examined.

Nevertheless, bacteriological standards are often imposed by the wholesalers. Table 1 indicates the standards now insisted on by some foreign purchasers on the Bulgarian market. So it would be necessary to look out for treatments in order to reduce contamination to an acceptable level. As in the case of spices we can consider a treatment with ethylene oxide. Another possibility would be a low dose irradiation completed by a mild heat treatment. We have tried this combined treatment in the following two cases: a preparation of dry onion soup and a liver paste. It seems unrealistic to achieve a regular sterilization, as the product is incorporated into unsterilized food.

Table 1  
*Bacteriological standards required by various utilizers  
of onion powder*

Organisms \ Factories	A	B	C
Total count/g	100 000	100 000	100 000
Coliform	100	100	—
<i>E. coli</i>	0	10	0
<i>Salmonella</i>	0	0	—
Hemolytic microorganisms	—	500	—
Spore-formers	10 000	—	—
Spore-formers producing starch hydrolysis	1 000	—	—
Anaerobic sulfite reducers	—	500	—
Yeasts	—	—	50
Molds	—	—	50

## 1. Materials and methods

The same sample of homogenized onion powder has been used from the beginning to the end of these tests. It is an industrial preparation obtained from yellow skin onions kept in cold silos before drying.

Irradiation is carried out with a  $^{60}\text{Co}$  source giving a dose rate of 0.4 Mrad/h, the temperature of the samples during irradiation being 25 °C.



The enumeration of microorganisms is carried out from a dilution containing 1 g of onion powder per 10 ml of 0.1 *M* pH 7.0 phosphate buffer. Either the technique of the most probable number or that of plating is used.

The culture medium used for the total count of mesophiles contains: meat extract 3 g, Bactotryptone 3 g, yeast extract 2 g, dextrose 1 g/l litre. pH = 7.2–7.4. Counting is done after an incubation of 120 hours at 30 °C.

Anaerobic spore-formers are determined after heating the sample during 10 min at 80 °C and inoculating in the special liquid medium M 11 containing beef heart recommended by the "Microbiological specifications and testing methods for irradiated food" (ANON, 1970). The incubation is done at 37 °C during 10 days.

The anaerobic sulfite reducers are characterized in the same media, adding after heating to each tube of 10 ml:

sodium sulfite, 7 H <sub>2</sub> O 5%	0.25 ml
ferrous ammonium citrate 5%	0.10 ml

## 2. Results

### 2.1. Microflora inactivation by irradiation

The onion powder irradiated in presence of air is rapidly sanitized with a dose of 0.4 Mrad. When the dose is increased up to 0.8 Mrad, the radio-resistant fraction of the microflora is not destroyed. Table 2 indicates the decrease of the total mesophile count. Fifteen colonies of different morphology have been isolated from the plate counting of the powder irradiated at 0.4, 0.6, 0.8 Mrad. They have been identified with a series of specific culture media as *Bacillus subtilis*, *B. firmus*, *B. pumilus*, *Streptococcus faecalis*, *Micrococcus roseus* and *M. varians*.

Table 2  
*Reduction of bacterial contamination by  
irradiation of onion powder*

Dose Mrad	Number of experiments	Total count per g	Spore-forming anaerobes	Spore-forming anaerobes, sulfite reducers
0	5	69 000	230	20
0.2	2	2 800		
0.4	4	460	17	2
0.6	2	350		
0.8	2	310		



The survival curve of a strain of *Str. faecalis* and *M. roseus* is plotted in Fig. 1. It has been obtained with a suspension of young cells in phosphate buffer irradiated in presence of air. In these conditions the above two strains are not particularly radioresistant; the decimal reduction doses (DRD) are 0.145 Mrad for *Str. faecalis* and 0.3 Mrad for *M. roseus*. The last figure is lower than the DRD of 0.55 Mrad obtained by DAVIS, SILVERMAN and MASUROVSKY (1963) with radioresistant pigmented coccus isolated from irradiated haddock.

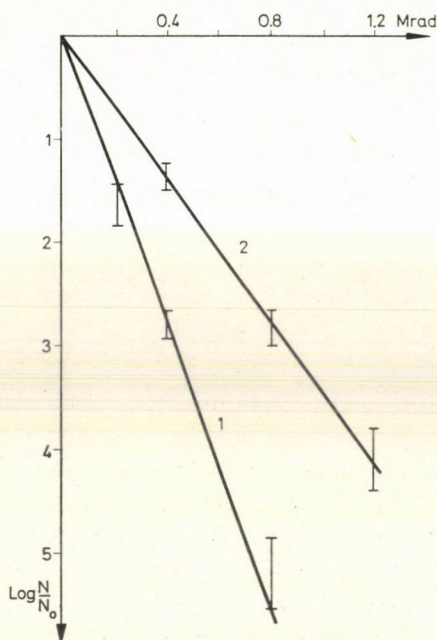


Fig. 1. Survival curve of two radioresistant strains isolated from irradiated onion powder

$$1: \textit{Str. faecalis} \frac{14-0.6}{1}; \quad 2: \textit{M. roseus} \frac{15-0.8}{3}$$

Anaerobic spore-formers are less sensible to irradiation than mesophilic bacteria. With a dose of 0.4 Mrad, 7% of the anaerobes proves to be resistant, whereas only 0.7% of the total flora behaves in a similar way. After irradiation counting of anaerobic sulfite reducers becomes difficult as they are few in number. We have verified that in these conditions of culture, onion powder at a concentration of 1% does not inhibit the growth of these bacteria.

During a 4-month's period of conservation of irradiated onion powder on repair of the damaged bacteria was noted, but a decrease of the revivable germs approximately identical with the one observed in non-irradiated powder (Fig. 2).

## 2.2. Heating of irradiated onion powder

We wanted to know whether supplementary reduction of the revivable germs could be obtained by subsequent heating. The results of Table 3 indicate that heating 80 min at 70 °C has only a small effect as to reducing the number of bacteria in non-irradiated onion powder. The fraction of the flora surviving a 0.2 Mrad irradiation seems quite resistant to this subsequent treatment. On the other hand, temperature cannot be increased above 70 °C, as it produces caramelization of the sugars, which gives a new product.

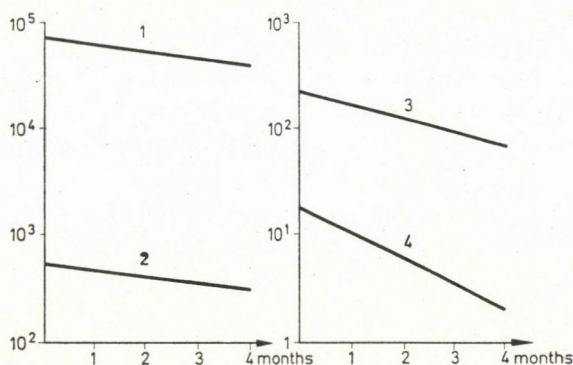


Fig. 2. Spontaneous reduction in number of viable cells of the natural flora in irradiated and non-irradiated onion powder. Storage temperature 22 °C. 1 and 3: non irradiated; 2 and 4: 0.4 Mrad irradiated; 1 and 2: total count, cells/ml; 3 and 4: anaerobic spore-formers, cells/ml

Table 3

*Effect of subsequent heat treatment at 70 °C on irradiated onion powder*

Heating time	Total count per g	
	non-irradiated	0.2 Mrad irradiation
0 min	44 000	1 200
20 min	—	700
40 min	40 000	700
80 min	13 000	1 000

## 2.3. Contamination of soup by sanitized and non-sanitized onion powder

A commercial dry soup "Double poule au vermicelle Knorr" is prepared according to instructions given on the packet and subsequently sterilized in flasks. We have added 1% of irradiated and non-irradiated onion powder, respectively, as follows:



- sample 1) non-irradiated powder,  
 2) 0.4 Mrad irradiated powder,  
 3) non-irradiated powder, boiled for 5 min,  
 4) 0.2 Mrad irradiated powder, boiled for 5 min,  
 5) 0.4 Mrad irradiated powder, boiled for 5 min.

The boiled samples are cooled in water. All the samples are incubated at 27 °C. The evolution of the microflora is shown in Table 4. We note that in the sample No. 1 which was neither heated, nor irradiated, only part of the inoculated organisms survived. According to calculation we ought to have found 680 germs per ml, whereas we have found only 137 per ml. As indicated by JOHNSON and VAUGHN (1969) this is due to the bactericidal disulfides liberated by enzymatic action on rehydrating the powder.

Table 4

*Evolution of the total count of bacteria in a commercial soup inoculated with 1% onion powder*

Irradiation dose of onion powder, Mrad	Boiling time of soup, min	Time, days									
		0		1		2		3		5	
		Total count per ml	pH	Total count per ml	pH	Total count per ml	pH	Total count per ml	pH	Total count per ml	pH
—	—	137	6.1	$25 \cdot 10^7$	4.5	$34 \cdot 10^7$	4.3	—	—	$22 \cdot 10^7$	4.0
0.4	—	2	6.1	$1 \cdot 10^5$	6.1	$44 \cdot 10^7$	4.2	—	—	$38 \cdot 10^7$	4.2
—	5	1	6.1	$5 \cdot 10^3$	6.1	pellicle	6.4	—	—	—	—
0.2	5	0	6.1	0	6.1	$9 \cdot 10^4$	6.1	pellicle	6.4	—	—
0.4	5	0	6.1	0	—	0	—	0	—	0	6.1

Irradiation at 0.4 Mrad alone permits to prolong the preservation of the soup by a day. In this case microbial alteration is produced by the same flora as in the case of non-irradiated powder. The main component is the radioresistant acidifying germ *Str. faecalis*, followed by the development of molds.

In the case of onion powder heat-treated by boiling alteration is produced, without acidification, by the development of *B. subtilis*. Previous irradiation of 0.2 Mrad delays the alteration. Combining a 0.4 Mrad irradiation with heating for 5 min at 100 °C we obtain a bacteriologically stable product.

#### 2.4. Contamination of liver paste by sanitized and non-sanitized onion powder

An industrial canned pork paste is contaminated with 1% onion powder non-irradiated and irradiated, respectively at the level of 0.2 Mrad. The product is distributed in glass jars and cooked in the oven. The jars are heated



slowly for 1 h 30 min, so as the center reaches 90 °C. This mode of heating roughly reproduces the usual cooking in the preparation of pastes.

After cooling at room temperature the jars are capped and incubated at 24 °C during 8 days to allow estimation of the microbial development. We remark in Table 5 that simple heating is not sufficient to secure the microbiological stability of the paste. It is spoiled by an aerobic flora of *B. subtilis*; just a few *Clostridium* begin to appear after 8 days.

Table 5  
*Contamination of liver paste inoculated  
with 1% onion powder*

Treatments	Bacterial count per g after	0	3	8 days
Uncontaminated control	Total count	0-10	0-10	0-10
	Anaerobes	0	0	0
Unirradiated onion powder	Total count	2-10	30-50	4-6 · 10 <sup>7</sup>
	Anaerobes	0	0	10-20
0.2 Mrad irradiated onion powder	Total count	0-10	0-10	0-10
	Anaerobes	0	0	0

On the other hand irradiation with 0.2 Mrad of the onion powder is sufficient to obtain a stable product.

### 3. Conclusions

Seasonings, such as onion powder, are very often more contaminated than the preparations themselves they are added to. Hence, they produce bacterial spoilage in the products which are not sterilized or necessitate an improvement of sterilizing conditions. Previous irradiation of the onion powder considerably reduces this contamination and makes possible to prepare bacteriologically stable products as we have shown in the cases of dehydrated soup and pastes.

When combining heat and irradiation treatments, it should be made sure that the order of the sequence is well chosen. In the case of spores of *Clostridium* or *Bacillus* it is well established that irradiation should precede heating (GRECZ *et al.*, 1967). However, it is not a general rule for all the microorganisms, as shown by SOMMER *et al.* (1967) for the conidia of *Cladosporium herbarum*, *Monilia fruticola*, *Botrytis cinerea* and *Penicillium expansum*. The same has been found by BALKAY *et al.* (1969) for the vegetative cells of yeast. In the case of onion powders it is not possible to carry out heating prior to irradiation, for the powder quickly turns brown and the



enzyme essential for the flavour development is inactivated. Besides, it is not advantageous to apply dry heat before irradiation, as we have shown that heating the dry powder is ineffective as to reducing bacterial count.

In the case of onion powders combined treatment of irradiation plus heat can be successfully utilized. It gives a means of preparing bacteriologically stable products. This has been established in the preparation of dehydrated soup and liver paste, hence these promising results suggest that the study of onion powder can be included in the programme of spices irradiation actually in progress (VAN DIJCK, 1970).

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## HEAT-RADIATION COMBINATION FOR CONTROL OF MOLD INFECTION IN HARVESTED FRUITS AND PROCESSED CEREAL FOODS\*

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A combination of mild heat and low dose irradiation was found to extend the shelf-life of fresh fruits and processed cereal foods by controlling mold infection. *Chapaties* (Indian unleavened bread) and bread slices packed in polycell pouches, subjected to 50 krad followed by dry heat (65 °C) were free from mold and shelf-stable for 10 weeks at ambient temperature (28—32 °C). Inoculated pack studies confirmed the efficacy of the treatment. No immediate changes in organoleptic attributes were discernible even after exposure to 100 krad. The quality deterioration in sliced bread stored for 2 1/2 months has been attributed to natural staling rather than radiation.

Hot water dip (50 °C for 5 min) followed by 150 krad irradiation extended the shelf-life of fresh figs by 3—4 days at 28—32 °C and 8—10 days at 15 °C. Regardless of the sequence of treatments, combination of heat and 100 krad extended the shelf-life of grapes both at ambient and refrigerated storage. In mangoes, heat followed by 50 krad was effective in controlling anthracnose and stem-end rot whereas in bananas irradiated for delayed ripening, hot water treatment can be used as a supplementary process to control stem-end rot. Quality of combination treated fruits was comparable to normally ripened fruits.

*In vitro* studies with fungal pathogens isolated from the above fruits and cereal foods revealed that the synergistic effect of heat-radiation combination depends on the sequence of treatments which varied with respect to different pathogens studied. Some biochemical aspects of combination treated fruits will be discussed.

Unlike chemical fungicides, gamma irradiation, due to its extreme penetration, can be used for treating deep seated pathogens within the host tissues to provide a therapeutic effect. However, the dose required for effective control of pathogens invariably results in undesirable changes in the host tissues. It is now generally recognized that the most promising means of increasing the utility and effectiveness of radiation in the control of fungal spoilage of fruits without adversely affecting the normal quality attributes, is to combine it with heat (ECKERT & SOMMER, 1967). Such combined treatments are more effective than either radiation or heat alone, the effects being more than additive. This has been shown in investigations with bacteria (HUBER *et al.*, 1953), fungi (SOMMER *et al.*, 1967) as well as preinoculated fruits (BEN-ARIE & BARKAI-GOLAN, 1969) and fruit juices (DHARKAR, 1964;

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DHARKAR *et al.*, 1966). Lowering the radiation dose as much as possible is desirable not only for wholesomeness, but also from the economic point of view.

Several investigators have demonstrated the usefulness of a combination of mild heat and low dose irradiation to extend the shelf-life of fresh fruits like strawberries (SOMMER *et al.*, 1968), citrus (AHMED *et al.*, 1969), pears (BEN-ARIE & BARKAI-GOLAN, 1969), peaches and nectarines (SOMMER *et al.*, 1967). While maximum attention has been given to disease control in fruits of temperate zone, relatively little work has been reported in case of tropical and sub-tropical fruits. STEHLIK and KAINDL (1968) have reported that bread slices could be kept for a long time free from mold attack by combination of dry heating and low dose irradiation.

In the present investigation attempts have been made to study the use of heat-radiation combination for control of mold infection in processed cereal foods like bread slices and *chapaties* (Indian unleavened bread) and in tropical and sub-tropical fruits like mangoes, bananas, figs and grapes. Fungal pathogens isolated from the above fruits and cereal foods, were used to study the synergistic effect of heat-radiation combination on the cultured isolates.

## 1. Materials and methods

### 1.1. Preparation of chapaties

The *chapaties* were prepared as described earlier (SAVAGAON *et al.*, 1970). Freshly milled, sifted flour (1 000 g), hydrogenated oil (100 g), water (600 ml) and salt (10 g) were mixed in a plate and kneaded to a soft dough which was divided into 50 parts. These were made into flat discs of about 6" diameter with a rolling pin and baked on a hot plate. The final product, ready to eat *chapaties*, had a moisture of 27–28%. The average composition of the finished product was dry flour 64.3, water 27.5, fat 7.4 and salt 0.8%.

A well-known brand of bread slices from wrapped packages were removed and, after air infection, packed in suitable packaging material and used subsequently for experiments.

### 1.2. Packaging of bread slices and chapaties

Three available packaging films, *i.e.*, polyethylene, polycell (polyethylene lining to cellophane) and paper, polyethylene, aluminium foil laminates, were tested for their suitability for packaging bread slices and *chapaties*. Polyethylene bags on irradiation acquired intensive radiation odour and imparted off odour to the product. Paper(foil)polyethylene laminated pouches



were unacceptable due to their off-odour and non-transparency. Hence, polycell pouches were selected because they are transparent, impermeable to bacteria, less permeable to gases and produce relatively less off-odour on irradiation. Water-vapour transmission rate of polycell film was found to be 1.62 g per sq. meter per 24 hour at 37 °C and at 80% relative humidity (SAVAGAON *et al.*, 1972).

### 1.3. Irradiation

*Chapaties* and bread slices, sealed two in each polycell bag, were exposed to gamma rays in a Food Package Irradiator (Atomic Energy of Canada Ltd., Commercial Products; 81 000 curies) at a dose rate of 300 krad per hour. The absorbed radiation dose was measured by Fricke's dosimeter (FRICKE & MORSE, 1927) and a ceric sulphate dosimeter.

### 1.4. Heat treatment

Dry heating of bread slices or *chapaties*, preceded or followed by irradiation treatment was carried out in an oven (or dryer) with 0.5 °C variation in temperature. The rate of heat penetration in bread slices and *chapaties* was measured with a thermocouple. Treated *chapaties* and bread slices along with untreated samples were stored at room temperature (28°–32°) in cardboard boxes. A minimum of ten packs (20 bread slices or 20 *chapaties*) were used for each treatment and repeated at least thrice.

### 1.5. Inoculated pack studies

Bread slices or *chapaties* were inoculated at four different spots with 0.2 ml spore suspension (about  $10^6$  spores/ml) of a mixture of *Aspergillus niger* and *A. flavus oryzae* and packed in polycell pouches as described earlier. The sealed packs were incubated at 30 °C for 24 hours and subsequently subjected to the various treatments.

### 1.6. Peroxide value

Finely cut *chapaties* and bread slices (50 g) were extracted in cold with analytical grade chloroform (100 ml) for 18–20 hours and 25 ml of the extract was taken for peroxide determination by the method of ROCKWOOD *et al.* (1947). Results are expressed as meq per kg of material.



### 1.7. Experiments with fruits

Figs, grapes, mangoes and bananas were procured from the local market. Fruits were normally 1–2-day-old from the time of harvest. Only fruits, free from apparent injury and of uniform size and maturity, were used. Two commercially important varieties each of mangoes (Alphonso and Langda), grapes (Anab-e-Shahi and Seedless) and bananas (Cavendish and Mysore) and in case of figs the variety Rajewadi were used.

### 1.8. Irradiation and hot water dip

Fruits packed in perforated polyethylene bags were irradiated in the Food Package Irradiator. Green mature mangoes (pre-climacteric stage) were irradiated with 25 and 50 krad. Cavendish and Mysore bananas in the preclimacteric stage were irradiated with 35 and 25 krads, respectively, the optimum doses for delaying ripening of these varieties. Grapes and figs of table ripe stage were irradiated with 100 and 150 krad, respectively.

Hot water dip at  $50^{\circ} \pm 0.2^{\circ}\text{C}$  for 5 min was given to the fruits in precision controlled water bath. Immediately after hot dip treatment, fruits were cooled for 5 minutes in a tank with running water at room temperature. Fruits were drained, blotted with tissue paper and stored in perforated cardboard boxes at ambient or  $15^{\circ}\text{C}$  temperatures. Equal number of fruits in case of mangoes, bananas and figs and equal quantity (by weight) of grapes were taken for the various treatments. All experiments were carried out at least in duplicate.

Daily observations were made on incidence of mold infection in fruits and cereal products.

### 1.9. Artificially inoculated fruits

Healthy fruits were artificially inoculated by dipping them in a slurry made from infected fruits (spore counts between  $10^3$ – $10^4$ /ml slurry) for two minutes and incubating at ambient temperature for 24 hours. These infected fruits were subsequently treated with combination of irradiation and hot water dip.

### 1.10. Organoleptic evaluation

Triangular tests for the evaluation of taste, texture and flavour of fruits as well as of cereal products, were carried out by 6–10 judges on a nine-point hedonic scale. *Chapaties* were warmed on a hot plate to about  $75^{\circ}\text{C}$  before

they were served to the taste panel. Grapes and figs were served immediately after treatments, whereas mangoes and bananas were taken for organoleptic evaluation after the fruits were fully ripe.

### 1.11. *In vitro* studies with spores of fungal pathogens

Fungi associated with the spoilage of the above commodities were isolated from naturally infected fruits and stock cultures were maintained. Some of the fungi of the *Aspergillus* group, isolated from bread and *chapaties*, were tentatively identified as *A. niger*, *A. terreus*, *A. flavus* (aflatoxin producing) and *A. flavus-oryzae*. *Rhizopus* sp. and *Aspergillus* sp. were the predominant spoilage organisms of figs, grapes and mangoes, respectively.

Spores of some of the isolated fungi, grown on potato dextrose agar (PDA) were harvested after 10 days with sterile distilled water containing a few drops of Tween-80. Mycelial fragments were removed by passing aseptically through 8 layers of cheese cloth. The spores were washed thoroughly with sterile distilled water and suspended in saline. Aliquots of 3 ml (about  $1.5 \times 10^6$  spores/ml) in sterile glass test tubes were irradiated in a gamma cell 220 (Atomic Energy of Canada Ltd., 22 500 curies) at an average dose rate of 110 krad per hour. Heat treatments were carried out before or after irradiation. For studying colony forming ability of the treated spores, aliquots of 1 ml were diluted according to the estimated percentage of spore germination and plated on PDA medium in a Petri dish. The developing colonies were counted microscopically after incubating for 24 hours at 25 °C and, again, after three and five days, in order to designate colonies which showed retarded development. Each treatment was carried out at least in 6 replicates.

## 2. Results

### 2.1. *Bread slices and chapaties*

Irradiation alone substantially brought down spoilage in bread and *chapaties* and increased the shelf-life of these cereal products. The extension in shelf-life of these products increased with increasing radiation dose (Fig. 1). At doses of 500 krad and above, *chapaties* and bread slices could be stored for two months or more at ambient temperature. Effects of heat treatment to varying degrees on incidence of mold on bread slices and *chapaties* are presented in Fig. 2. Heating the *chapaties* and bread slices up to 65 °C for 35 minutes reduced the incidence of mold infection. Compared to irradiation or heating alone, a combination of heat and low dose irradiation remarkably delayed the spoilage of these products (Table 1). Results also indicated that



Table 1

*Mold incidence in bread slices and chapaties during storage at ambient temperature (28°–32 °C)*

Treatment	% spoilage												
	Bread slices						Chapaties						
	Days of storage												
	2	5	7	10	15	30	60	5	7	10	15	30	60
No treatment*	30	80	100	—	—	—	—	50	80	100			
50 °C + 50 krad	—	10	50	60	60	80	100	20	40	70	80	100	
50 krad + 50 °C	—	—	10	20	40	60	100	10	30	40	60	80	100
60 °C + 50 krad	—	—	—	10	30	40	60	—	10	30	40	60	100
50 krad + 60 °C	—	—	—	10	20	40	40	—	—	10	20	60	80
65 °C + 50 krad	—	—	—	—	—	20	40	—	—	—	—	10	30
50 krad + 65 °C	—	—	—	—	—	—	—	—	—	—	—	—	—
50 °C + 100 krad	—	—	10	20	40	100	—	—	10	20	30	80	100
100 krad + 50 °C	—	—	—	10	30	60	100	10	30	40	40	80	100
60 °C + 100 krad	—	—	—	—	—	—	30	—	—	—	—	20	40
100 krad + 60 °C	—	—	—	—	—	10	10	—	—	—	—	20	20

\* For each treatment 10 packs were used. Subsequent heat treatments were given for 35 minutes. A combination of 50 krad and heating (65 °C, 35 min) completely inactivated spoilage fungi in bread slices and chapaties.

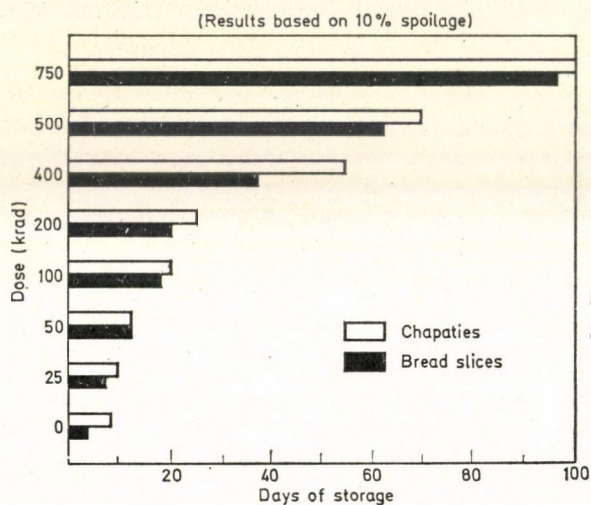


Fig. 1. Extension of storage life of bread and chapaties by gamma rays. At doses above 500 krad both bread slices and chapaties could be stored at room temperature (28–32 °C) for more than two months free of fungal attack

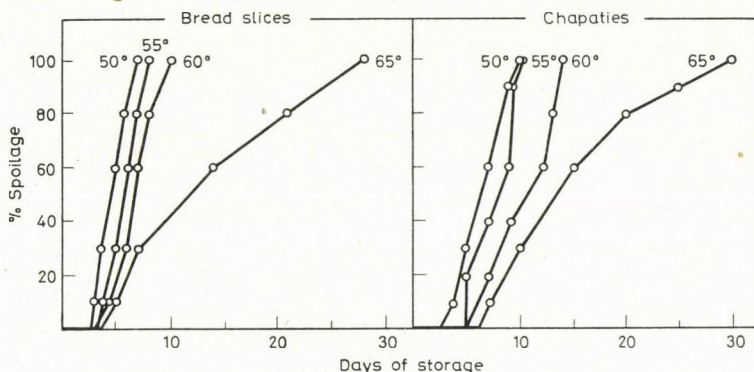


Fig. 2. Effect of heating on incidence of mold in bread slices and chapaties. Dry heating to 65 °C for 35 min delayed the incidence of mold

heating after irradiation was more effective than heating before irradiation. Optimum heating temperature was found to be 65 °C and radiation dose can be as low as 50 krad. Bread and *chapaties* subjected to the above combination treatment remained free from mold infection for a period of two months. If the radiation dose is doubled (100 krad) followed by heating to 60 °C for 35 minutes, bread and *chapaties* can be stored for over a month without mold infection.

The effectiveness of combination treatment was further revealed from inoculated pack studies (Fig. 3), where 50 krad dose followed by heating

Table 2

*Effect of heat-radiation combination on quality attributes of bread slices and chapaties stored at room temperature*

Product	Storage days	Moisture* content %	Peroxide* value meq/kg	Organoleptic** score
Bread slices (without treatment)	0	39.1	0.15	7.3
Bread slices (with 50 krad + 65 °C, 35 min)	0	38.8	0.16	6.9
	30	37.1	0.23	4.5
	60	35.9	0.28	3.9
Chapaties (without treatment)	0	27.8	0.25	8.15
Chapaties (with 50 krad + 65 °C, 35 min)	0	27.15	0.24	8.2
	30	25.5	0.29	6.5
	60	23.8	0.35	6.0

\* Average of 5 values.

\*\* Based on 1–9 hedonic scale. Scores above 5 indicate acceptable and below 5 not acceptable. Chapaties were acceptable even after 2 months of storage while bread slices were not acceptable due to staling.



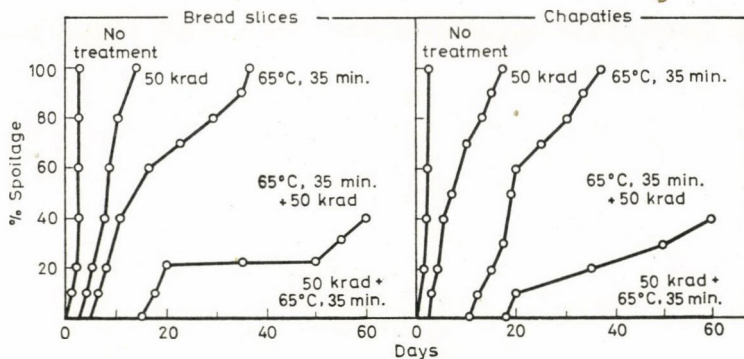


Fig. 3. Effects of combination treatment on artificially inoculated bread slices and chapaties. A combination of 50 krad followed by heating to 65 °C 35 min totally inactivated the preinoculated fungal spores of *Aspergillus niger* and *A. flavus oryzae*

Table 3

Effect of heat-radiation combination on spoilage of mangoes at ambient temperature (28°–32 °C)

Variety/treatment	Per cent infected fruit										
	Days of storage										
	6	8	10	12	14	16	18	20	22	24	26
<b>ALPHONSO</b>											
Nil	—	—	20	20	40	40	60	100			
Heat (50 °C, 5 min)	—	—	—	—	20	20	40	60	60	80	100
25 krad	—	—	—	10	10	30	40	40	60	80	100
50 krad	—	20	20	40	40	60	80	100			
Heat + 25 krad	—	—	—	—	—	—	10	20	20	40	60
Heat + 50 krad	—	—	—	—	—	—	—	20	20	40	40
25 krad + Heat	—	—	—	—	20	20	40	50	70	80	100
50 krad + Heat	—	—	—	—	—	20	20	40	40	60	100
<b>LANGDA</b>											
Nil	10	20	40	50	90	100					
Heat (50 °C, 5 min)	10	20	20	40	70	90	100				
25 krad	20	40	40	70	80	100					
50 krad	10	20	20	40	70	80	100				
Heat + 25 krad	—	—	10	10	20	40	50	50	50	70	90
Heat + 50 krad	—	—	—	10	20	20	40	40	60	60	60
25 krad + Heat	10	20	20	50	70	90	100				
50 krad + Heat	10	10	30	50	60	80	100				

Alphonso mangoes used in these experiments were of green mature stage whereas Langda mangoes were of semi-ripe stage. A combination of heat (50 °C, 5 min) followed by radiation (50 krad) gave maximum reduction in spoilage.

to 65 °C for 35 minutes, could totally inactive the mold in *chapaties* and bread slices. Data on moisture loss, peroxide values and organoleptic score of chapaties and bread slices stored for 2 months are given in Table 2. Moisture content of bread slices and chapaties decreased with the storage period. Though peroxide values showed a gradual increase with storage, this did not result in rancidity of the products. Both products, while served fresh immediately after combination treatment, were highly acceptable. *Chapaties*, even after two months of storage, were acceptable; however, bread slices were unacceptable, which could be attributed to staling.

## 2.2. Fruits

Data on heat-radiation combination on spoilage of different varieties of mangoes, bananas, grapes and figs are shown in Tables 3, 4, 5 and 6, respectively. The form of heat treatment employed in these experiments was a

Table 4

*Effect of heat-radiation combination on spoilage of bananas  
(28°–32 °C temperature storage)*

Variety/treatment	Per cent infected fruit								
	Days of storage								
	4	5	6	7	8	9	10	11	12
<b>MYSORE</b>									
Nil	10	30	80	100					
Heat (50 °C, 5 min)	—	—	—	—	—	10	20	40	60
25 krad	50	80	100						
Heat + 25 krad (5 min)	—	—	—	10	30	50	100		
Heat + 25 krad (10 min)	—	—	—	—	—	10	20	50	80
25 krad + Heat (5 min)	—	10	20	50	90	100			
25 krad + Heat (10 min)	—	—	10	40	50	80	100		
<b>CAVENDISH</b>									
Nil	—	—	—	10	50	100			
Heat (50 °C, 5 min)	—	—	—	—	—	10	20	40	100
35 krad	—	—	10	30	100				
Heat + 35 krad (5 min)	—	—	—	—	—	10	40	80	100
Heat + 35 krad (10 min)	—	—	—	—	—	—	10	50	100
35 krad + Heat (5 min)	—	—	—	—	10	30	80	100	
35 krad + Heat (10 min)	—	—	—	—	—	10	50	80	100

Fruits were subjected to the various treatments in the pre-climacteric stage. A combination of heating and radiation delays ripening as well as incidence of rot.



Table 5

*Effect of heat-radiation treatments*

Treatment	Per cent				
	20°—32 °C; RH 60—75%				
	Days of				
	1	2	3	4	5
Nil	60	100			
Heat (50 °C, 5 min)*	20	50	100		
100 krad	30	50	100		
150 krad	10	40	100		
Heat + 100 krad	—	—	10	40	100
Heat + 150 krad	—	—	10	40	80
100 krad + Heat	30	50	100		
150 krad + Heat	20	50	80	100	

\* Heating (50 °C, 5 min) followed by 150 krad was more effective than vice versa.

Table 6

*Effect of heat-radiation combination*

Variety/treatment	Per cent infected							
	28°—32 °C; RH 60—75%							
	Days of							
	4	6	8	10	12	14	16	18
<i>ANAB-E-SHAHI</i>								
Nil	10	40	90	100				
Heat (50 °C, 5 min)	—	10	40	75	100			
100 krad	20	40	80	100				
Heat + 100 krad	—	—	—	10	20	50	100	
100 krad + Heat	—	—	20	30	40	75	100	
<i>SEEDLESS</i>								
Nil	2	20	50	100				
Heat (50 °C, 5 min)	—	6	30	70	100			
100 krad	2	5	25	50	100			
Heat + 100 krad	—	—	—	—	10	25	30	50
100 krad + Heat	—	—	2	5	10	25	50	100

Regardless of the sequence, combination of heat and irradiation extended the irradiation is slightly more effective than hot water dip after irradiation.

*on incidence of mold in figs*

infected fruit (weight)										
15 °C; RH 70–80%										
storage										
1	2	3	4	5	6	7	8	9	10	11
30	50	100								
10	30	50	100							
—	30	60	100							
—	—	10	50	80	100					
—	—	—	—	—	—	—	10	30	100	
—	—	—	—	—	—	—	—	10	70	100
—	—	—	10	30	100					
—	—	10	30	50	100					

*on spoilage of grapes*

fruit (weight)											
15 °C; RH 70–80%											
storage											
6	8	10	12	14	16	18	20	22	24	26	28
10	30	70	100								
—	—	10	30	50	100						
5	10	30	50	100							
—	—	—	—	—	5	10	20	50	100		
—	—	—	—	5	10	30	30	100			
5	10	40	100								
—	—	—	—	10	25	40	60	100			
20	30	50	60	100							
—	—	—	—	—	—	—	—	—	—	5	10
—	—	—	—	—	—	—	2	5	10	50	100

shelf-life of grapes both at ambient and refrigerated temperatures. Hot water dip before



mild hot water dip at 50 °C. In Alphonso and Langda mangoes, the maximum tolerable radiation dose was found to be 75 krad, whereas for Cavendish and Mysore bananas, it was only 35 krad. It was also observed that these fruits could not tolerate heat treatment above 55 °C. Heating above 55 °C resulted in scalding and, in extreme cases, abnormal ripening. It can be seen that a combination of heat and irradiation delayed mold incidence in mangoes and bananas stored at ambient temperature (28–32 °C) (Tables 3 and 4). Heating prior to irradiation seems to be particularly beneficial to mangoes where combination with 50 krad gave maximum fungal control.

Figs and grapes are usually harvested at table ripe stages and thus are more susceptible to rot. Normal shelf-life of figs and grapes at ambient temperature is 1–2 and 4–6 days, respectively (Tables 5 and 6). So far as changes in quality attributes are concerned, figs and grapes seem to be relatively resistant to large doses of radiation. No appreciable flavour or textural changes were observed even after figs and grapes had been irradiated with 150 and 100 krad, respectively. Both fruits could also reasonably tolerate

Table 7

*Extension of shelf-life of artificially inoculated fruits after treatment of optimum combination of gamma rays and mild hot water dip*

Fruit	Treatment	Storage period in days		Organoleptic score*
		28–32 °C	15 °C	
Figs	No treatment	1	2	6.75 (7.0)
	Heat + 150 krad	4	8	
Grapes	No treatment	2	8	7.10 (7.30)
Anab-e-Shahi	Heat + 100 krad	10	18	
Grapes	No treatment	4	9	7.70 (7.85)
Seedless	Heat + 100 krad	14	22	
Mangoes	No treatment	10	—	8.10 (8.50)
Alfonso	Heat + 50 krad	26	—	
Mangoes	No treatment	8	—	7.50 (7.83)
Langda	Heat + 25 krad	16	—	
Bananas	No treatment	7	—	7.0 (7.33)
Cavendish	Heat + 35 krad	10	—	
Bananas	No treatment	4	—	7.9 (8.5)
Mysore	Heat + 25 krad	9	—	

Values are based on a 20 per cent wastage basis.

\* Grapes and figs were served to trained panel members immediately after treatment, whereas mangoes and bananas were served when they reached eating stage. Figures in parentheses represent fresh, ripe, untreated fruits.

hot water dip at higher temperatures. Heating followed by irradiation at 150 krad extended the shelf-life of figs by 3–4 days at 28°–32 °C and 8–10 days at 15 °C (Table 5). It is also seen from the table that irradiation, heating or refrigeration alone did not retard the fungal development appreciably. Regardless of the sequence of treatments, combination of heat and 100 krad extended the shelf-life of grapes both at ambient and sub-room temperature storage, hot water dip before irradiation showing a slight edge over hot water dip after irradiation. Anab-e-Shahi grapes were more prone to rot than Seedless.

All the four species of fruits were artificially inoculated with slurries of corresponding infected fruits and subjected to the combined heat-irradiation treatment, optimum for each fruit. There was considerable delay in development of mold in these fruits both at ambient and sub-room temperature storage (Table 7). Such combination treated fruits were comparable to untreated fruits at their best in respect of organoleptic attributes.

### 2.3: *In vitro* studies with fungi isolated from cereal products and fruits

2.3.1. *Fungi of bread and chapaties*. Results on the radiosensitivity of spores of the *Aspergillus* group, isolated from *chapaties* and bread slices, are presented in Fig. 4. From the results it seems that *A. niger* is the most resistant strain, whereas *A. flavus oryzae* is the most sensitive strain. However,

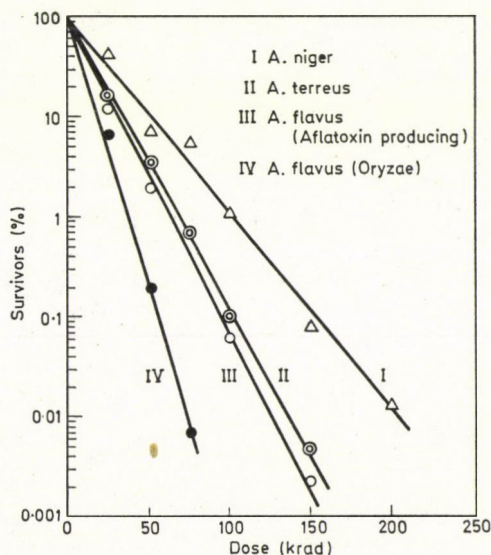


Fig. 4. Approximate dose response curves for fungi of the *Aspergillus* group isolated from bread and *chapaties*. *A. niger* was most resistant to radiation, whereas *A. flavus oryzae* was the most sensitive among the four species studied



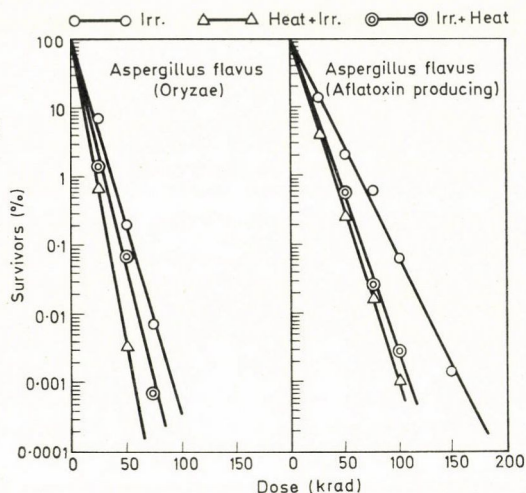


Fig. 5. Effect of heat-radiation combination on the survival of fungal spores. Heat-radiation sequence was more effective in case of both the species of *Aspergillus*

noticeable variation in germinating and colony forming ability of these fungi was observed, when spores were subjected to a combination of heat and irradiation (Figs. 5, 6). Maximum sensitization of both the strains of *A. flavus* (aflatoxin producing and non-toxic) occurred when heating preceded irradiation, whereas in *A. niger*, heating after irradiation was more effective. In *A. terreus*, the sequence of treatments did not show any appreciable difference in their synergistic effect.

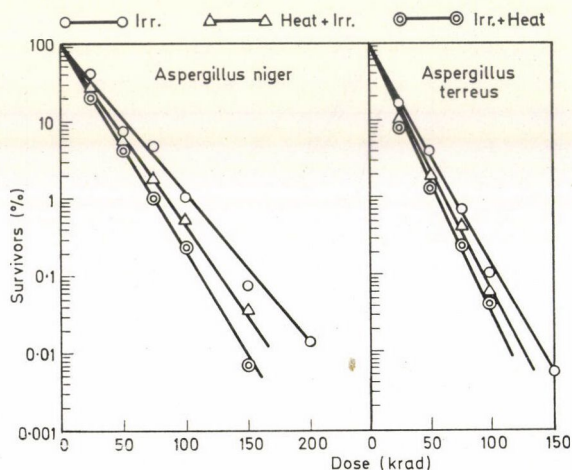


Fig. 6. Effect of heat-radiation combination on the survival of fungal spores. In case of *A. niger* radiation-heat sequence was more effective whereas in *A. terreus* the sequence of treatments did not show any appreciable difference in their synergistic effect

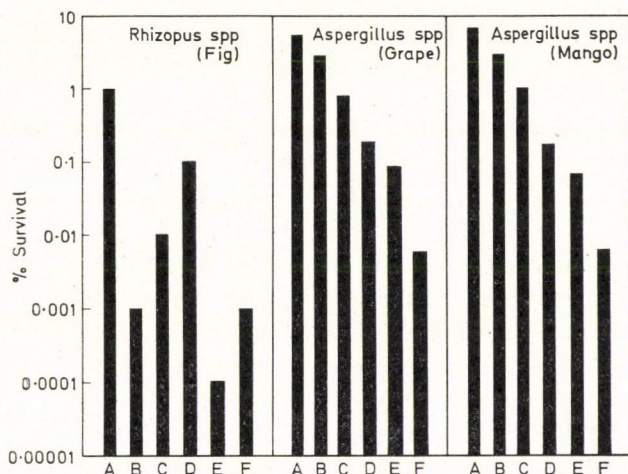


Fig. 7. Survival of fungal spores after combined treatment of heating and irradiation. A — 50 krad; B — Heat (50 °C, 5 min) + 50 krad; C — 50 krad + 50 °C, 5 min; D — 100 krad; E — 50 °C, 5 min + 100 krad; F — 100 krad + 50 °C, 5 min

2.3.2. *Fruit pathogens.* *Rhizopus* and *Aspergillus* species, isolated from figs, grapes and mangoes, were studied *in vitro* for synergistic effects of combination of heat and irradiation. Results are shown in Fig. 7. Combinations of heat + irradiation sequence seem to be more effective with *Rhizopus* spp. *Aspergillus* species isolated from grapes as well as mangoes, were more susceptible to irradiation + heat sequence.

### 3. Conclusions

The results for *chapaties* and bread slices are in agreement with the earlier findings from this laboratory for *chapaties* (SAVAGAON *et al.*, 1970) and for bread slices from other laboratories (STEHLIK & KAINDL, 1968). However, the dose required (500 krad) for shelf-life extension of *chapaties* and bread slices is high. Although there are reports to show that wheat irradiated at doses much higher than those required for insect disinfestation is nutritionally wholesome, larger doses of irradiation induce unacceptable changes in quality attributes of these products. Combination of low dose irradiation and heat treatment appears to be more advantageous to extend the shelf-life of bread slices and *chapaties* at ambient temperature. At present there are no effective methods to preserve *chapaties* neither at ambient nor at sub-room temperatures. Use of a chemical preservative reduces the organoleptic qualities of *chapaties* (KAMESWAR RAO *et al.*, 1964). KAMESWAR RAO *et al.* (1966) claimed that *chapaties* could be stored longer if 4% salt or 1.5% salt



and 0.3% sorbic acid were incorporated into the dough. However, from the acceptability point of view, incorporation of salt is not desirable. Bread can be preserved for almost any length of time at low temperatures at which essentially all microbial activity is arrested (OTTAWAY, 1958). However, this method has only limited application. Heat treatment can be used for sterilization of packaged bread. SCHULZ (1955) showed that, by one hour heating of packaged bread to 90–100 °C, mold development is delayed by at least 8 weeks. In another investigation (OTTAWAY, 1958), unsliced bread loaves were packaged in cellulose film and subjected to a temperature of 180 °C for 45 minutes. However, with all these treatments, mastication of the bread becomes difficult and the product becomes unacceptable. Packaging materials could also impart off-odour to the bread. STEHLIK and KAINDL (1968) observed that even canned bread slices were not acceptable. In the present studies, though the bread slices subjected to combination of irradiation and heat were not acceptable as such after a two-month storage on account of staling, they could be used after toasting.

Both varieties of mangoes are susceptible to anthracnose and stem-end-rot diseases, which normally develop at a later stage of ripening. Several fungicides have been tried to control these diseases in Alphonso and Langda mangoes (SUBRAMANYAM *et al.*, 1972; NAURIYAL *et al.*, 1972). Hot water dip, alone or in combination with chemical fungicides, has been shown to be effective in delaying fungal incidence in mangoes (PENNOCK & MALDONALDO, 1962; SMOOT & SEGALL, 1963; THOMAS & DALAL, 1968). However, heat treatment augments ripening, thereby reducing total shelf-life. Hot water dip combined with low doses of irradiation thus seems a better process in that delay both in ripening and fungal infection can be achieved. In bananas, hot water dip can be used as a supplementary process to control stem-end-rot, since hot water dip alone could delay the occurrence of stem-end-rot to a considerable extent in Cavendish and Mysore bananas (Table 4). Even though a number of investigators have tried different fungicides, an effective control of this predominant rot of bananas, is yet to be achieved (MEREDITH, 1971). It was also observed that the optimum doses necessary for delaying the ripening process in Cavendish and Mysore bananas (35 and 25 krad, respectively), accelerate the development of stem-end-rot which could be delayed by combining irradiation with hot water dip. Fig pathogens seem preferentially sensitive to heat-irradiation sequence. Effective heat-irradiation sequence for control of fruit pathogens has also been reported in other fruits like peaches and nectarines (SOMMER *et al.*, 1967), pears (BEN-ARIE & BARKAI-GOLAN, 1969), citrus (AHMED *et al.*, 1969), strawberries (SOMMER *et al.*, 1968). In India, about 50% of the total harvested figs are spoiled due to fungal rot. There is no effective measure to control this rot, since the fruits have very short shelf-life at ambient or even sub-room temperatures of storage. The



higher sugar content of grapes makes them more vulnerable to fungal attack. Irradiation alone incidentally accelerates rotting in grapes. Grapes could be stored at low temperatures ( $0^{\circ}$ – $5^{\circ}\text{C}$ ) for longer periods without fungal attack; however, at ambient or sub-room temperatures, combination of heat-irradiation can delay the rotting in grapes, thereby extending the shelf-life.

Results of *in vitro* studies on fungi isolated from cereal products indicate that the synergistic effect of mild heat and low-dose radiation depends on the sequence of treatments which varies with respect to different fungal pathogens. In case of certain bacteria, irradiation followed by heating is more effective (KEMPE, 1955). Our observations on fruit pathogens are similar with respect to those which have been obtained for *Penicillium expansum*, *Botrytis cinerea* and *Alternaria tenuis* (BEN-ARIE & BARKAI-GOLAN, 1969), *Monilinia fruticola* and *Cladosporium herbarium* (SOMMER et al., 1967) and *Penicillium digitatum* (BARKAI-GOLAN et al., 1969). SOMMER et al. (1967), however, observed that radiation + heat sequence was more effective in case of *Rhizopus stolonifer* isolated from stone fruits.

The observations that effectiveness of combination processing depends upon the sequence of the treatments for specific organisms, is difficult to explain. The spores of *A. flavus*, treated with heat + irradiation combination, fail to recover even after 4 days of incubation in distilled water with aeration, whereas, in case of spores irradiated only (up to 100 krad dose), considerable recovery was observed. Similar results were obtained in *A. niger* sp. with irradiation + heat treatment (PADWAL-DESAI et al., 1972). Irradiation and heating are physical treatments in which mode and site of action may be different. The damage to cellular DNA is one of the major factors responsible for radiation lethality, whereas heating inactivates proteinaceous materials in the cell by denaturing them. Cells have an ability to rejoin DNA strand breaks and this is one of the most important factors governing susceptibility of cells to radiation (SZYBALSKI, 1966). The rejoining of DNA strand breaks is a complex process involving several enzyme systems like endonuclease, exonuclease, DNA-polymerase and polynucleotide ligase (SZYBALSKI, 1967). Effectiveness of irradiation in combination with mild heating can be explained on the basis of inactivation of an enzyme or enzymes concerned with DNA repair. Experiments are in progress to understand some of the biochemical events governing the effects of heat and radiation combination on sensitization of fungi.

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## THE ROLE OF HEAT RESISTANCE IN THERMORESTORATION OF HYDRATED BACTERIAL SPORES\*

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This study for the first time presents evidence of the distinct role played in thermorestitution by cellular determinants such as the resistance to heat and radiation, and the ionic state of spores. In the past only radiochemical determinants associated with radical annealing have been studied in hydrated systems. The basic heat resistance of spores plays a significant role in the precipitous drop in spore survival due to 0.45 Mrad radiation plus heat above 65—75 °C for *B. cereus* and 75—95 °C for *B. stearothermophilus*. The effect of the spores' radiation resistance was not distinct except in the frozen state and at the saturation plateau of thermorestitution where the radiation resistant *B. cereus* showed ca. 1 log cycle higher survival than the radiation sensitive *B. stearothermophilus*. When spores are chemically converted into their  $H^+$  and  $Ca^{++}$  ionic forms, the  $H^+$  spores are distinctly more responsive than  $Ca^{++}$  spores to processes of radical annealing responsible for thermorestitution in hydrated spore systems. At temperatures of extensive thermorestitution of water radicals,  $H^+$  spores showed higher survival than  $Ca^{++}$  spores.

This study for the first time presents evidence of the distinct role played in thermorestitution by cellular determinants such as the resistance to heat and radiation and the ionic state of spores. In the past only radiochemical determinants associated with radical annealing have been studied in hydrated systems.

The term thermorestitution is used to describe an apparent increase in survival (of bacterial spores) exposed to combinations of simultaneous radiation and heat at gradually increasing temperature levels above 0 °C (SUCHANEK *et al.*, 1969). Previously this phenomenon was termed "paradoxical inversion of radiation sensitivity of spores" (WEBB *et al.*, 1958), however, subsequently it was recognized that the processes responsible for increased survival of spores involved restoration by temperature of radiation induced radicals. Therefore "thermorestitution" appeared to be a more appropriate term.

From an analysis of studies in our laboratory, of aqueous systems a typical thermorestitution survival pattern can be divided into at least 6 phases (Fig. 1).

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Phase 1. Frozen state below 0 °C; spores show high radiation survival because radicals are trapped in ice.

Phase 2. Highly lethal conditions around 0 °C where radicals are mobile and abundant.

Phase 3. Rapid change in radical annealment rates between 0° and ca. 15 °C.

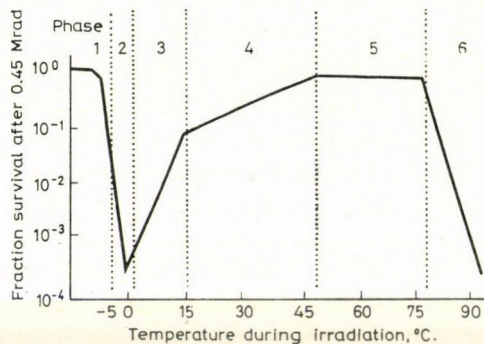


Fig. 1. Thermorestoration survival pattern

Phase 4. Slow-down due to saturation of annealment rates (or perhaps saturation of spore susceptibility) above ca. 20 °C.

Phase 5. Level portion due to complete saturation of thermorestoration processes above ca. 50 °C.

Phase 6. Highly lethal terminal range above 65–75 °C for *B. cereus* and above 75–95 °C for *B. stearothermophilus*.

Dry spores show a similar survival pattern except that the point of lowest survival occurs at 37 °C (POWERS, 1965).

The specific thermorestoration survival pattern is the result of both environmental and cellular determinants. With respect to environmental determinants a strict distinction has been made between (i) *dry spores* in defined gaseous environments or in vacuum (WEBB *et al.*, 1958, 1960; POWERS *et al.*, 1959, a, b; POWERS, 1965; POWERS & TALLENTIRE, 1968; TALLENTIRE & POWERS, 1963) and (ii) hydrated spores in aqueous environments including water, buffer, ground meat and broth (EDWARDS *et al.*, 1954; KEMPE *et al.*, 1956, 1959; GRECZ *et al.*, 1963, 1967b; GRECZ, 1965, 1966; FRIEDMAN, 1972).

Radicals formed by radiation in dry spores in vacuum can be affected by O<sub>2</sub> (sensitization), or NO, H<sub>2</sub>O, heat (protection.) Although the radicals in the spores which react with these agents have not yet been identified, they were characterized on the basis of their apparent properties by POWERS and co-workers (POWERS, 1965; POWERS & TALLENTIRE, 1968). Damage from these radicals has been assigned into three classes: Class I direct hit damage

essentially independent of radioprotective and radiopotentiating agents; Class III long-lived radicals which can become lethal by reaction with  $O_2$ , or harmless by reaction with  $H_2O$  vapour, with NO or by heating after or during irradiation (thermorestoration), and Class II, due to very reactive short lived radicals which normally dissipate during irradiation but can be converted into lethal species if  $O_2$  is present during irradiation.

It should be noted that dry thermorestoration is restricted to Class III damage exclusively. Since dry thermorestoration could be eliminated with  $H_2O$ -vapour, it is obvious that thermorestoration observed in fully hydrated spores in aqueous media involves processes different from those of Class III. These considerations suggest also that there are at least two (and possibly more) distinct mechanisms leading to thermorestoration.

Dry anoxic spores sustain radiation damage from direct hits, while hydrated spores in distilled water sustain essentially 99% of their damage from indirect action *via* radiolysis products of water (GRECZ *et al.*, 1967a). In this context it is important to review briefly the essential features of radiochemistry of water involved in wet thermorestoration (Fig. 2). Under anaerobic conditions, the main damaging radical responsible for indirect action is thought to be the hydroxyl radical (JOHANSEN, 1965; JOHANSEN & HOWARD-FLANDERS, 1965; BLOCK *et al.*, 1967). The hydrated electron and the hydrogen radical seem to play no significant role in biological damage. In the presence of oxygen all radicals found in anoxic water also occur, however, the hydrated electron as well as the hydrogen radical react with oxygen as shown in Fig. 2 and are converted into bactericidal species of relatively long half-life, the peroxy and the perhydroxyl radical. The presence of  $O_2$  in water (i) increases the net yield of biologically harmful radicals and (ii) causes production of additional species which are not formed under anoxic conditions, *viz.* the peroxy ( $\cdot O_2$ ) and perhydroxyl ( $\cdot HO_2$ ) radicals. In addition,

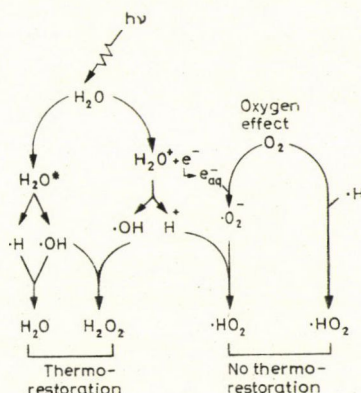


Fig. 2. Radiolysis products of water as affected by thermorestoration. Biological toxicity of radicals in decreasing order:  $\cdot OH > \cdot HO_2 > H_2O_2 > \cdot H, e^-_{aq}$



oxygen may possibly react directly with radical species in the spore producing lethal lesions as was found in dry systems.

Elevated temperatures such as encountered in thermorestitution enhance recombination of  $\cdot\text{OH} + \cdot\text{H}$  radicals, but they do not remove harmful species due to  $\text{O}_2$ , *i.e.* neither  $\cdot\text{HO}_2$  radicals nor any other products of  $\text{O}_2$  causing lethal damage (SUCHANEK *et al.*, 1969; GRECZ *et al.*, 1969).

As evident from this review, environmental determinants of thermorestitution are relatively well known, however inherent (or imposed) cellular determinants have not been investigated to date. The purpose of the present project was to elucidate the role of cellular properties of spores affecting their thermorestitution survival in aqueous systems. The following properties were considered: (i) basic heat resistance; (ii) basic radiation resistance and (iii) chemically modified  $\text{H}^+$  and  $\text{Ca}^{++}$  ionic forms of spores.

## 1. Materials and methods

### 1.1 Bacterial strains

Wild type *Bacillus cereus* T from Dr. H. O. Halvorson, University of Minnesota; and *B. stearothermophilus* NCIB 8224 from Dr. J. Tramer, United Dairies Ltd., London, W. 12 were selected on the basis of their heat and radiation resistances. Stock cultures were maintained on nutrient agar slants.

For *B. stearothermophilus*, the presporulation medium for propagation of the initial inoculum contained tryptone, 1%; yeast extract (Difco), 0.5%; and  $\text{K}_2\text{HPO}_4$ , 0.2% (TYG); pH 7.2 before autoclaving (KIM & NAYLOR, 1966).

The sporulation medium contained nutrient broth (Difco) 0.8%; yeast extract (Difco), 0.4%;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 10 ppm; Difco agar, 2.0%; pH 7.2 before autoclaving (KIM & NAYLOR 1966), dispensed into 15×150 mm plastic Petri dishes (Falcon Plastics); usually 80 to 100 ml of agar was used.

For *B. cereus*, the presporulation medium was trypticase soy broth (Difco). The sporulation agar containing tryptone, 0.5%; yeast extract, (Difco), 0.25%; glucose, 0.15%; Difco agar, 1.5%; pH 7.0, was dispensed into 15×150 mm plastic Petri dishes (Falcon Plastics) and allowed to solidify.

### 1.2. Spore production and cleaning: *Bacillus stearothermophilus*

A loopful of the stock culture was transferred to a 25 ml Erlenmeyer flask containing 10 ml of presporulation media (TYG). The broth was incubated on a New Brunswick rotary shaker for 14 hours. A 2-ml aliquot from this broth was then spread onto the surface of one 15×150 mm Petri dish containing the sporulation agar. This plate was incubated in an upright position at 55 °C overnight. When the agar surface appeared to be dry, the plate was inverted



and incubated at 55 °C until maximum sporulation had occurred. After approximately 96 hours of incubation, 75% of the cells had sporulated (determined by microscopic examination). In order to produce larger volumes of spores, growth from a sporulation agar plate was carefully washed off with 20 ml of TYG broth and added to 80 ml of additional TYG broth, which was next incubated on a rotary shaker for 14 hours at 55 °C. This broth was used to seed 25, 15 × 150 mm Petri dishes of sporulation agar, about 2 ml of culture per plate. The plates were incubated in an upright position and after 96 hours at 55 °C, 75% sporulation had occurred. The plates were cooled to room temperature and all growth was washed off each plate with 20 ml of ice cold distilled sterile water per plate. The washings from each plate were transferred to a flask immersed in crushed ice. The resulting 500 ml of spore suspension was centrifuged in a refrigerated centrifuge to sediment the spores. After decanting the supernatant fluid, an equal volume of cold distilled water was added to resuspend the sediment (MOLIN & SNYGG, 1967).

For cleaning of spores, lysozyme was added as a 1% solution at the rate of 1 ml per 100 ml of spore suspension. Overnight refrigeration at 4 °C resulted in the liberation of all spores from sporangia and the lysis of unsporulated vegetative cells. The spore suspension was then washed 10 times by centrifugation, decanting the supernatant fluid, and resuspended in equal volumes of sterile ice-cold distilled water.

### 1.3. Spore production and cleaning: *Bacillus cereus*

A loopful of the stock culture of this organism was transferred to 25 ml of Trypticase soy broth in a 250-ml Erlenmeyer flask. The culture was incubated at 30 °C for 18 hours. This broth was then used to seed Petri dishes containing the sporulation agar (HALVORSON, 1957). The plates were first incubated in an upright position until no fluid was observed, they were then inverted and allowed to incubate until maximum sporulation had occurred as observed microscopically. After 72 hours incubation approximately 95% of all cells sporulated (WALKER *et al.*, 1961).

After complete sporulation, all plates were refrigerated for 18 hours to aid in the lysing of the remaining vegetative cells. The growth on agar surfaces was then washed off with ice-cold sterile distilled water and the spores were treated with lysozyme and washed in the manner described for cleaning of *B. stearothermophilus*.

### 1.4. Preparation of spore chemical states: *B. stearothermophilus*

The heat sensitive state (hydrogen form) was prepared by suspending the untreated spores (10<sup>7</sup>/ml) in distilled water and adjusting to pH 3.5 with



1 *N* HCl. The acidified spore suspension was incubated at 70 °C for 1 hour then at 25 °C for 3 hours. By using a magnetic stirring bar the suspension was constantly mixed so that all spores would be uniformly sensitized. After 3 hours at 25 °C the suspension was adjusted to a pH of 6.5 with 1 *N* NaOH (ALDERTON & SNELL, 1969a, b).

Because of the tendency of spores to clump under acidic conditions, the spore suspensions were briefly mixed in a Waring Blender after both 70 °C and 25 °C incubation periods had been completed (LEWIS *et al.*, 1965).

The hydrogen form of the spores was returned to the resistant form (calcium form) by suspending the spores in 0.02 *M* calcium acetate which had been adjusted to pH 9.7 with aqueous Ca(OH)<sub>2</sub>, and incubating this suspension for 18 hours at 25 °C, followed by a 3 hour incubation at 80 °C. Again, both mixing and blending were utilized to facilitate uniform Ca<sup>++</sup> absorption and to prevent clumping. The resistant spores were then centrifuged, washed three times with distilled water, and stored in screw-cap Pyrex tubes at 4 °C (ALDERTON *et al.*, 1964).

#### 1.5. Preparation of spore chemical states: *Bacillus cereus*

Suspensions of *B. cereus* (10<sup>9</sup>/ml) in distilled water were converted to the resistant form by suspending the spores in 0.02 *M* calcium acetate adjusted to pH 8.0 and incubating at 60 °C overnight. The spores were then centrifuged and washed with distilled water and stored at 4 °C (ALDERTON *et al.*, 1964).

#### 1.6. Determination of spore concentration and spore viability

The number of spores was determined by direct microscopic count and by plate counts of serial dilutions. Aliquots of 2 ml of spore suspensions were heat-activated in sterile screw-cap tubes. Spores of *B. cereus* were brought to 80 °C for 10 minutes; while spores of *B. stearo-thermophilus* were subjected to a similar heat treatment, but at a temperature of 100 °C for 10 minutes in a constantly stirred ethylene glycol bath.

Decimal dilutions of heat shocked spores were made in sterile distilled water. One ml sample of each dilution was then transferred to 15×100 mm plastic Petri dishes. Spores of *B. cereus* were plated on Tryptone Glucose Yeast Extract Agar (Difco) and incubated for 48 hours at 30 °C. Spores of *B. stearo-thermophilus* were plated on Dextrose Tryptone Bromocresol Purple Agar (Difco) and incubated at 55 °C for 48 hours (MOLIN & SNYGG, 1967).

The number of colonies counted were averaged and expressed in terms of the number of viable spores per ml of original spore suspensions.



### 1.7. Determination of heat resistance

To determine the heat resistance of the spores, 1.2 ml of each of the spore suspensions were dispensed into 10 × 150 mm Pyrex test tubes. The suspension was then saturated with N<sub>2</sub> by bubbling the gas through each tube for 5 minutes and the tubes were sealed in an oxygen flame. Heating of the spores was done by total submerging of the sealed tubes in a water bath. Tubes were removed from the bath according to a pre-arranged experimental time schedule and immersed in a crushed ice water bath. In determining the thermal resistance of *B. stearothermophilus* a bath of ethylene glycol instead of water was used to achieve temperatures that exceeded 100 °C.

The control samples (no irradiation) were exposed to identical conditions as the irradiated samples (e.g. a come-up time of 2.5 minutes; a 7.4 minute exposure at each temperature; and a rapid cooling in crushed ice water bath, 3 minutes).

Survivors were measured as described in the previous section. Thermal death was defined as failure to grow into visible colonies on agar plates.

### 1.8. Irradiation of spores

Cleaned heat shocked spores were distributed in 1.2-ml aliquots into 10 × 75 mm Pyrex tubes.

The samples were kept in ice water during equilibration with nitrogen gas. Anoxic conditions were achieved in the spore suspension by bubbling nitrogen gas through the sample for 5 minutes. While the tube was flame sealed, gas was continually blown into the top of the tube in order to insure saturation of the sample (SUCHANEK *et al.*, 1969). The spores were irradiated with <sup>60</sup>Co to 0.45 Mrad. (Dose rate  $6.1 \times 10^4$  rad/min.) The temperature during irradiation was controlled at -15° to 120 °C in regularly spaced temperature intervals in 5° or 10 °C increments. Come-up time was 2.5 minutes, exposure time 7.4 minutes, cooling was 3 minutes. Temperature was maintained within  $\pm 2$  °C by a specially constructed blower box (GRECZ *et al.*, 1965). All irradiations were performed at the U. S. Army Natick Radiation Laboratory, Natick Massachusetts, by U. S. Army personnel. Radiation survival of spores was determined as described in the previous section.

## 2. Results

### 2.1. Heat resistance of selected organisms

As illustrated in Fig. 3, the hydrogen form of *B. stearothermophilus* showed a D value (time necessary to reduce spore counts by 90%) of 6 minutes



at 115 °C. The calcium form showed a D value of 15 minutes at 115 °C. Both curves are in agreement with the data of ALDERTON and SNELL (1963), indicating the  $\text{Ca}^{++}$  form spores to be considerably more resistant to heat than the hydrogen form.

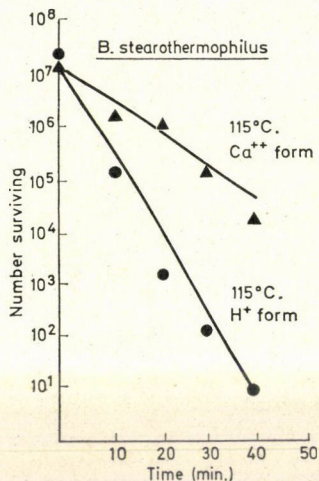


Fig. 3. Heat survival curves of chemically modified spores of *B. stearothermophilus* at 115°, evaluated by regression analysis using the method of "least squares" (SNEDECOR & COCHRAN, 1967)

The D values at 90 °C for *B. cereus* were 7 minutes for the hydrogen form, and 15 minutes for the  $\text{Ca}^{++}$  form (Fig. 4).

## 2.2. Radiation resistance

The highly heat resistant *B. stearothermophilus* showed a much lower resistance to gamma radiation with a  $D_{10}$  value (dose necessary to reduce spore counts by 90%) of 0.12 Mrads while the heat sensitive *B. cereus* exhibited a  $D_{10}$  value of 0.23 Mrads for the  $\text{H}^+$  form and 0.22 Mrads for the  $\text{Ca}^{++}$  form (Fig. 5). There were only slight differences in radiation resistance between spores in the heat sensitive  $\text{H}^+$  form and the heat resistant  $\text{Ca}^{++}$  form.

## 2.3. Heat resistance vs. thermorestitution: *B. stearothermophilus*

Radiation survival of the  $\text{H}^+$  form of *B. stearothermophilus* plotted as a function of temperature during irradiation (Fig. 6) shows the general features of the characteristic pattern associated with the thermorestitution of hydrated spores (GRECZ *et al.*, 1967b; SUCHANEK *et al.*, 1969). Spores had a relatively high radiation resistance in solidly frozen medium (Phase 1), followed by a



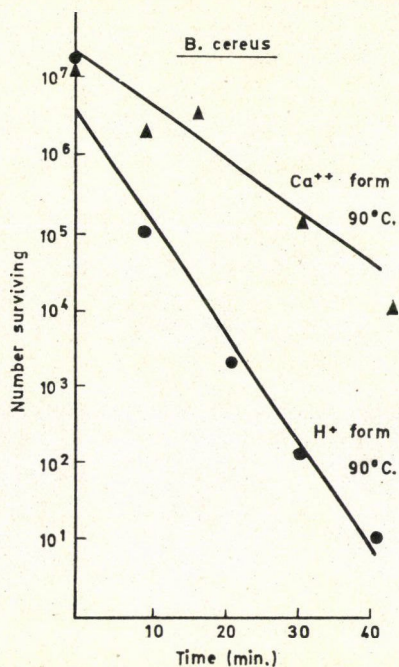


Fig. 4. Heat survival curves of chemically modified spores of *B. cereus* at 90 °C, evaluated by regression analysis using the method of "least squares" (SNEDECOR & COCHRAN, 1967)

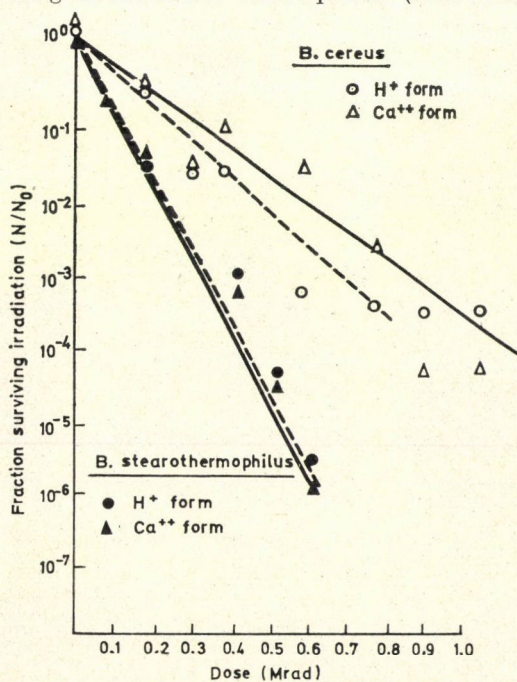


Fig. 5. Radiosurvival curves of *B. stearothermophilus* and *B. cereus* spores irradiated at 0 °C, evaluated by regression analysis using the method of "least squares" (SNEDECOR & COCHRAN, 1967)



sharp drop in the number of survivors during the transition from solid to liquid at 0°C (Phase 2). As temperature increased from 0°C to +5°C, survival increased by 2 to 2½ log cycles (Phase 3), between 5°C and 80°C survival increases by another 1 to 1½ log cycles (Phases 4 and 5). The alternate scatter of points seems to be due to experimental variations. At a maximum thermorestoration of 77–80°C, the curve indicates a greater than 3 log increase in spore survival over that achieved at 0°C. Above 80°C the survival declined precipitously (Phase 6) until at 110–120°C almost no survivors were detected. This drop above 80°C was consistent with a corresponding drop in the control curves receiving heat alone without radiation.

Fig. 7 represents the survival pattern of the  $\text{Ca}^{++}$  (heat resistant) form of *B. stearothermophilus* spores. Thermorestoration in Phases 1, 2 and 3 appeared to be identical to that of the  $\text{H}^+$  form spores. However, in Phases 4 and 5,  $\text{Ca}^{++}$  spores appeared to exhibit a greatly reduced sensitivity to the effect of thermorestoration processes. Starting at about 95°C (Phase 6) spore survival decreased extremely rapidly. Again the controls receiving no radiation indicated a corresponding drop commencing at about 100°C.

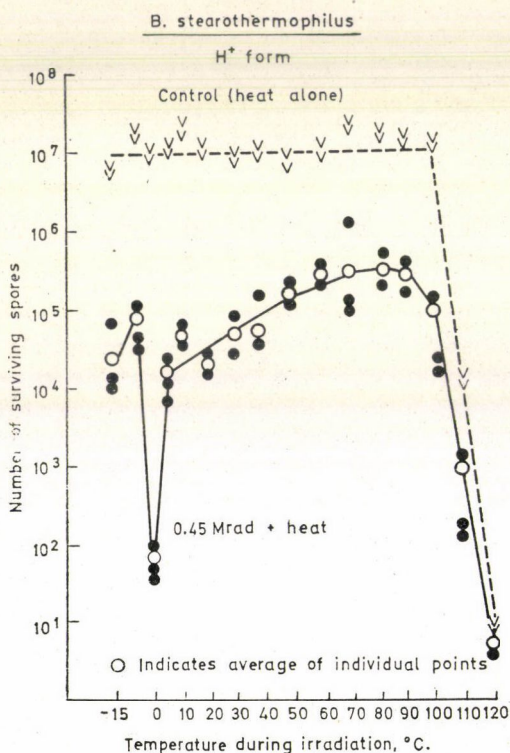


Fig. 6. Effect of temperature on radiation survival of *B. stearothermophilus* spores in the  $\text{H}^+$  form

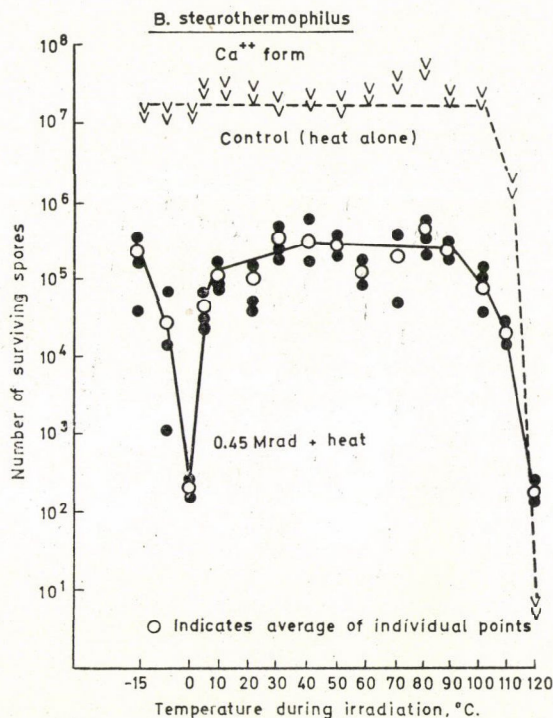


Fig. 7. Effect of temperature on radiation survival of *B. stearothermophilus* spores in the Ca<sup>++</sup> form

#### 2.4. Heat resistance vs. thermorestitution: *B. cereus*

Heat sensitive H<sup>+</sup> spores (Fig. 8) show high survival at subzero temperatures (Phase 1), but survival drops some 4 log cycles to a very low level at about 0 °C (Phase 2). The thermorestitution commences above 0 °C (Phases 3, 4 and 5) with over 4 log cycle increase between 0 °C and 65 °C. At about 65 °C the survival rate appears to have reached its maximum. Phase 6 sets in as the survival drops abruptly through 4–6 log cycles. The sharp decline in survival commencing at about 65 °C was paralleled by a corresponding drop in survivors at 70 °C in control spores receiving heat alone without radiation.

Survival of heat resistant Ca<sup>++</sup> spores of *B. cereus* (Fig. 9) resembles the pattern in Fig. 8 for H<sup>+</sup> spores except that the number of surviving spores of the Ca<sup>++</sup> form was throughout appreciably lower (by some one log cycle) than the H<sup>+</sup> form. The thermorestitution pattern of spore survival in all phases followed the classical picture including a typical well-expressed level plateau in Phase 5. Beyond about 70 °C the irradiated spores exhibited a sharp reduction (Phase 6) which again was paralleled by a corresponding drop in counts above 80 °C in control counts of spores receiving heat but no radiation.



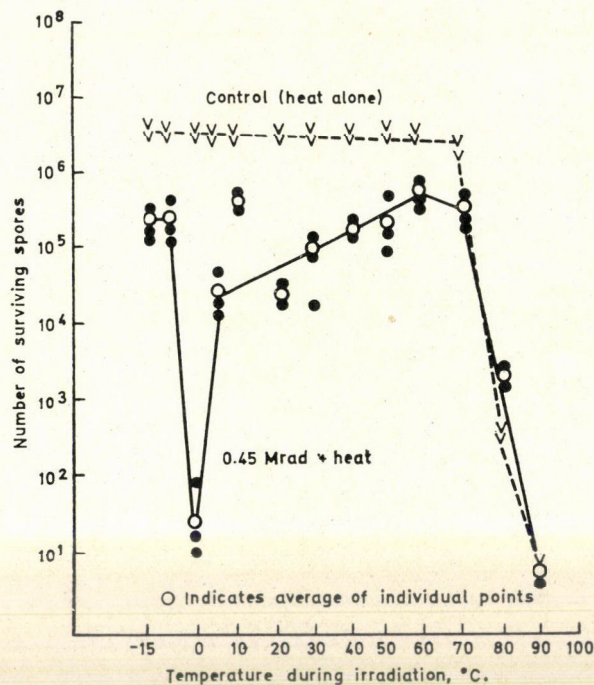


Fig. 8. Effect of temperature on the radiation survival of *B. cereus* spores in the  $H^+$  form

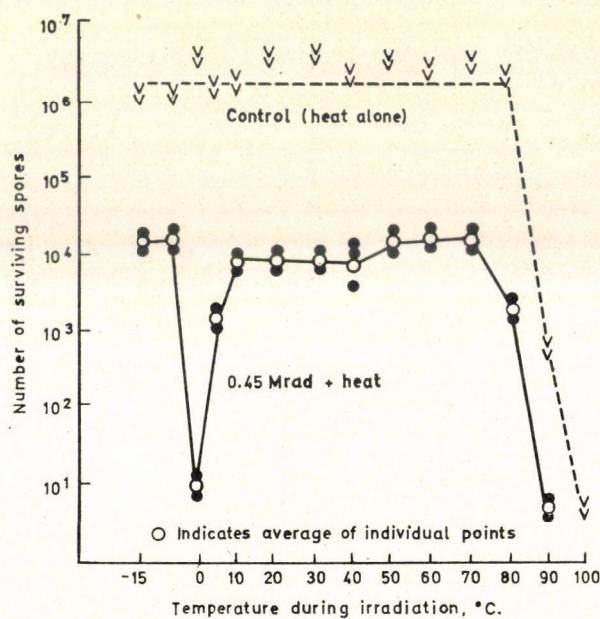


Fig. 9. Effect of temperature on the radiation survival of *B. cereus* spores in the  $Ca^{++}$  form



### 3. Conclusions

#### 3.1. Relation of spores' heat resistance to thermorestitution survival patterns

Fig. 10 shows that for both *B. cereus* and *B. stearothermophilus*,  $H^+$  form spores were clearly more responsive than  $Ca^{++}$  spores to processes underlying thermorestitution in Phases 3, 4 and 5, i.e.,  $H^+$  spores were probably more responsive to indirect action of ionizing radiation. The  $Ca^{++}$  spores were essentially as responsive as  $H^+$  spores in Phase 3 (0 °C to ca. 15 °C), but in Phase 4,  $Ca^{++}$  spores showed diminished responsiveness (*B. cereus*) or no responsiveness at all (*B. stearothermophilus*). Here again even in the  $Ca^{++}$  form, the heat sensitive *B. cereus* spores were more responsive to thermorestitution

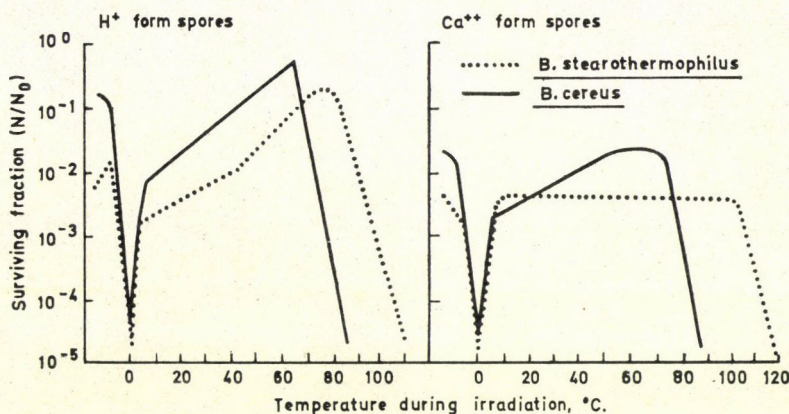


Fig. 10. Generalized thermorestitution curves of  $H^+$  and  $Ca^{++}$  spore forms of *B. cereus* and *B. stearothermophilus*

tion than the  $Ca^{++}$  spores of *B. stearothermophilus*, indicating that high heat sensitivity may be important. In Phase 5,  $Ca^{++}$  spores were non-responsive, in contrast to heat sensitive  $H^+$  spores which showed pronounced changes in survival levels in this phase.

The differences in heat resistance at 121 °C between the two species, *B. stearothermophilus* and *B. cereus* were ca. 2000 fold (MURRELL, 1955) and yet the difference in their responsiveness to thermorestitution was considerably less than that between  $Ca^{++}$  and  $H^+$  spores of the same species which differed in heat resistance only by ca. 2–3 fold (Figs. 3, 4). It must be concluded, therefore, that increased responsiveness to thermorestitution of the  $H^+$  form spores must be due to the  $H^+$  state itself and not to increased heat sensitivity. Water molecules in  $H^+$  form spores appear to be less strongly bound than in the  $Ca^{++}$  form spores (TJOA, HOFFMANN & GRECZ, 1973); this may suggest an explana-



tion for the high responsiveness of  $H^+$  form spores to thermorestitution, since free water in  $H^+$  spores may be essential for indirect action of radiation which plays a role in thermorestitution, while bound water in  $Ca^{++}$  spores may not participate in radiation effects.

### 3.2. Role of spore heat resistance in loss of viability in Phase 6

As shown in Table 1 the onset of precipitous kill of spores exposed to 0.45 Mrad plus heat (Phase 6) commenced at 75 °C and 95 °C for  $H^+$  and  $Ca^{++}$ , respectively, for *B. stearothermophilus*; and at 65 °C and 75 °C for *B. cereus*  $H^+$  and  $Ca^{++}$  forms, respectively. Table 1 also shows that the onset of the steep decline of viable spore counts in Phase 6 commenced at 3–5 °C lower temperatures for spores receiving radiation and heat, than those receiving heat alone. However, the difference between breakdown points of *B. cereus* vs. *B. stearothermophilus* was ca. 10 °C for  $H^+$  form spores and 20 °C for  $Ca^{++}$  form spores; this difference was the same for spores subjected to heat and radiation as for those subjected to heat alone. The conclusion seems obvious that the point of breakdown of spore resistance barrier at elevated temperatures with or without radiation was primarily due to the basic inherent heat resistance of spores.

Table 1  
Temperature (°C) of breakdown of heat resistance barrier\*

Spores of	Ionic form	Heat plus 0.45 Mrads radiation	Heat alone (control)
<i>B. stearothermophilus</i>	$H^+$	75	80
	$Ca^{++}$	95	100
<i>B. cereus</i>	$H^+$	65	68
	$Ca^{++}$	75	80

\* Data from Figs. 6, 7, 8 and 9.

### 3.3. Effect of radiation resistance

High radiation resistance of *B. cereus* appeared to have no other effect except increasing survival level by ca. 1 log cycle in Phases 1, 4 and 5; this effect was especially evident with  $H^+$  spores, but much less with  $Ca^{++}$  spores (Fig. 10). In Phases 2, 3 and 6 survival levels changed very extensively with change in temperature so that comparison of radiation resistant with radiation sensitive spores became impossible.



It may be premature to speculate on the mode of action of radiation on spores at the different temperatures, however, the possibility must be considered that at 0 °C DNA (attacked by  $\cdot\text{OH}$  radicals) and repair enzymes (attacked by  $\cdot\text{H}$  radicals) may both be affected and thus may contribute to increased spore death. At higher temperatures,  $\cdot\text{H}$  radicals may become exhausted by thermorestitution and thus DNA repair enzymes may not be destroyed.

Recently DURBAN (1971); DURBAN & GRECZ (1972, unpublished) have established that DNA single strand breaks produced in spores by radiation may be eliminated by direct repair occurring in spores immediately *during* irradiation involving DNA-ligase. The difference in radiation resistance of spores of the same species, like those tested in the present study, appears to be primarily due to activity of DNA-ligase in resistant spores and apparent lack of such activity in radiation sensitive spores.

#### 3.4. Quantitative analysis: contribution of radiation and heat to kill of spores

For comparison purposes the data presented in Figs. 6 and 7 for *B. stearothermophilus* and Figs. 8 and 9 for *B. cereus* have been recalculated to give factor  $F$

$$F = \frac{(N/N_0) \text{ for unirradiated controls (heat alone)}}{(N/N_0) \text{ for spores receiving radiation + heat}},$$

where  $N$  = number of spores surviving a particular treatment, heat alone or radiation + heat;  $N_0$  = initial viable number of spores in the sample.

For a combined simultaneous heat plus radiation treatment, the magnitude of factor  $F$  gives a quantitative index of the effect of radiation as distinguished from the effect of heat.

A value of  $F = 1$  would indicate no effect of radiation, *i.e.*, the effect of heat with radiation would be the same as heat without radiation. Any value of  $F > 1$  would characterize the contribution of radiation to spore kill, while  $F < 1$  constitutes the effect of heat alone.

#### 3.5. Results of calculations

The  $F$  factors calculated from our data are illustrated in Figs. 11 and 12. In all cases, the contribution of radiation to spore inactivation was highest around 0 °C, *i.e.*, in Phase 2. In Phases 1 and 4 through 5, the contribution of radiation to spore kill appeared to depend on the effects of temperature and solid-liquid phase transition on radical activity, *i.e.*, on indirect radiation effects. The effect of radiation decreased steadily in Phases 3–4,



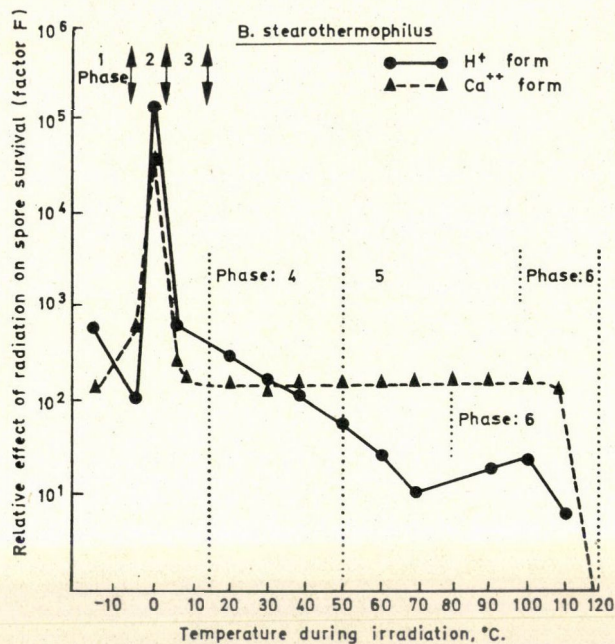


Fig. 11. Quantitative analysis of lethal actions of radiation on spores of *B. stearothermophilus* subjected to simultaneous radiation and heat

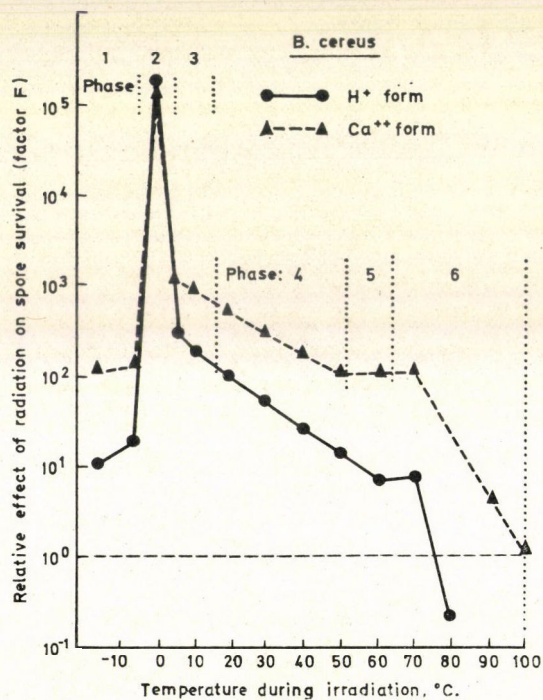


Fig. 12. Quantitative analysis of lethal actions of radiation on spores of *B. cereus* subjected to simultaneous radiation and heat



but this decrease became very slow and uncertain in Phases 4–5. During late Phase 6, at highly lethal temperatures a steep decline in F factors became apparent, although F did not become less than one except for the most heat sensitive spores of *B. cereus* H<sup>+</sup> form.

These calculations indicate that lethal heat effects became increasingly dominant in late Phase 6, however, the contribution of radiation to spore kill, although steadily diminishing was still definitely detectable since F was >1. The decline in contribution of radiation to spore kill in late Phase 6 may be the result of two opposing processes:

- (i) decreased activity of radiation radicals due to their extensive annealing and
- (ii) increased lethal action of heat alone at these high temperatures.

\*

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## PRELIMINARY STUDY CONCERNING THE INFLUENCE OF COMBINED HEAT AND RADIATION TREATMENT ON THE QUALITY OF SOME HORTICULTURAL PRODUCTS\*

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In collaboration with a canning factory a number of horticultural products were preserved by means of the combined irradiation and heating treatment. The aim was to study the influence of the parameters irradiation and heat on quality and microbiological spoilage.

The products were exposed to gamma-rays with doses varying from 0 to 1 Mrad. The heat treatment varied from 5 to 40 minutes at 98 to 121 °C; time and temperature depended on the type of product. The sterilization value ( $F$  value) was measured by means of the method of *Esty* and *Meyer* for products with  $\text{pH} > 5$ . The canned products were stored afterwards at 20—55 °C.

The organoleptic test showed that the colour and texture of most products were improved by the combined treatment, provided the irradiation dose did not exceed 0.25 Mrad. Strawberries in syrup discoloured at 0.1 Mrad. For a number of products the improved colour was partially lost during storage.

Flavour and taste were not generally influenced unfavourably at doses up to 0.3 Mrad (exception: strawberries).

The treatment sequence did not affect distinctly the quality or microbiological spoilage.

The sterilization time ( $F$  value) of the combined treatment was determined by the shortest heating period required for the tenderness of the product.

All preservation methods aim to maintain the original nutritional value and the organoleptic qualities of the food. An ideal preservation process should result in an unaltered stabilized fresh product.

The most used process up to date is heat treatment. During the last years food preservation by ionizing radiation was developed.

Either method had its advantages and drawbacks. Foods being bad heat conductors, require high temperatures and long sterilization times affecting their nutritional and organoleptic (colour, flavour, texture) properties.

Gamma rays as ionizing radiation possess a high penetrating power thus being capable to treat the product more homogeneously. Inactivation of the most resistant microorganisms has to be carried out at high doses. These generally cause unacceptable flavour and texture changes.

It is hoped that by a combination of both methods, at low irradiation dose and low  $F$  value, enzymes will be inactivated and the majority of

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the microorganisms eliminated, without the unwanted side effects of both methods.

Moreover, this combined treatment may lead to synergistic reactions resulting in stronger effect from this combination than would be expected from the sum of both treatments.

A number of authors has studied this effect. Generally sporulating microorganisms were made more heat sensitive by previous irradiation. KEMPE (1955) found a reduction of 3 for the radiation dose to inactivate *Cl. botulinum* and even a reduction of 4 for the heat treatment.

Bacterial spores gave no synergism with the sequence heat-radiation, vegetative cells, however, did (DUGGAN *et al.*, 1963, HANSEN & WARNØE, 1960).

Little to no attention has been paid to the quality aspects or to the technology while studying these combined treatments. In this paper the influence of the combined effect on the shelf-life and organoleptic properties will be discussed for six different horticultural products. Additionally the influence of the sequence of the combined treatment will be investigated (LANGERAK, 1967–1969).

## 1. Materials and methods

The products were industrially canned at a processing plant. For each variety of conditions of the combined treatment 10 cans were available. The *F* values were determined by the ESTY and MEYER (1922) method for products with  $\text{pH} > 5$ .

The pasteurization scheme for strawberries and pears in syrup ( $\text{pH} < 5$ ) accorded with the theoretical organism calculations of 1 min at 85 °C at a *z* value of 10.

Before irradiation the heat treated cans were cooled to 20 °C after filling to prevent quality losses.

The quality was assessed by a lab/consumer panel. The scale 1 to 10 was used (1 very bad, 10 excellent). The samples were tested versus the regularly processed items as control. Test data were calculated according to KRAMER (1956, 1960).

The texture of some samples was measured by means of the tendrometer (DOESBURG, 1960).

The samples were irradiated at the Pilot Plant for Food Irradiation, Wageningen, the Netherlands ( $^{60}\text{Co}$  source of 105 kCi, dose rate 6 krad/min, 10% accuracy) 1 to 3 hrs after heat treatment.

Microbiological control of the products was carried out by incubation at 20, 37 and 55 °C. Products with a  $\text{pH} > 5$  were tested for

- a) mesophilic anaerobic spores in a reinforced clostridial medium (Oxoid M 149),
- b) mesophilic aerobic spores in a nutrient agar (Oxoid CM<sub>3</sub>),
- c) thermophilic aerobic spores in a dextrose tryptone agar (Oxoid CM 75).

Details concerning all experimental treatments can be found in the legends of tables and figures.

## 2. Results

### 2.1. Strawberries in syrup

From a preliminary experiment it was concluded that the treatment sequence was not important. Hence only the sequence heat-irradiation was investigated. All tests were carried out with 0.5-l cans. The control was pasteurized at 20 min 98 °C, resulting in an *F* value of 3.2 min/85 °C.

The experimental samples were pasteurized at 10 min 98 °C, resulting in an *F* value of 0.9 min/85 °C.

After six months storage the strawberries were tested for colour, flavour, taste and texture.

Fig. 1 demonstrates an improved colour, taste and texture for the shorter pasteurization times. Increasing doses give a diminishing improvement. Over 0.1 Mrad the organoleptic quality became debatable.

The observed radiation sensitivity of the natural colouring agents necessitated the use of an artificial agent (Ponceau 4R) (see Fig. 1).

The effect of a short pasteurization time and irradiation on the texture as measured with the tenderometer is shown in Table 1.

These measurements indicate that the improved texture is better demonstrated by a physical method than in an organoleptic test.

Table 1  
*Tenderometer values (8-kg spring) of strawberries in syrup  
stored 1 year at 20 °C*

Heat + Irradiation		
<i>F</i> value + Dose		Firmness (kg)*
3.2 min/85 °C + 0	Mrad	1.2
0.9 min/85 °C + 0	Mrad	3.2
0.9 min/85 °C + 0.05	Mrad	3.3
0.9 min/85 °C + 0.075	Mrad	1.5
0.9 min/85 °C + 0.1	Mrad	1.3

\* Average of 2 measurements.



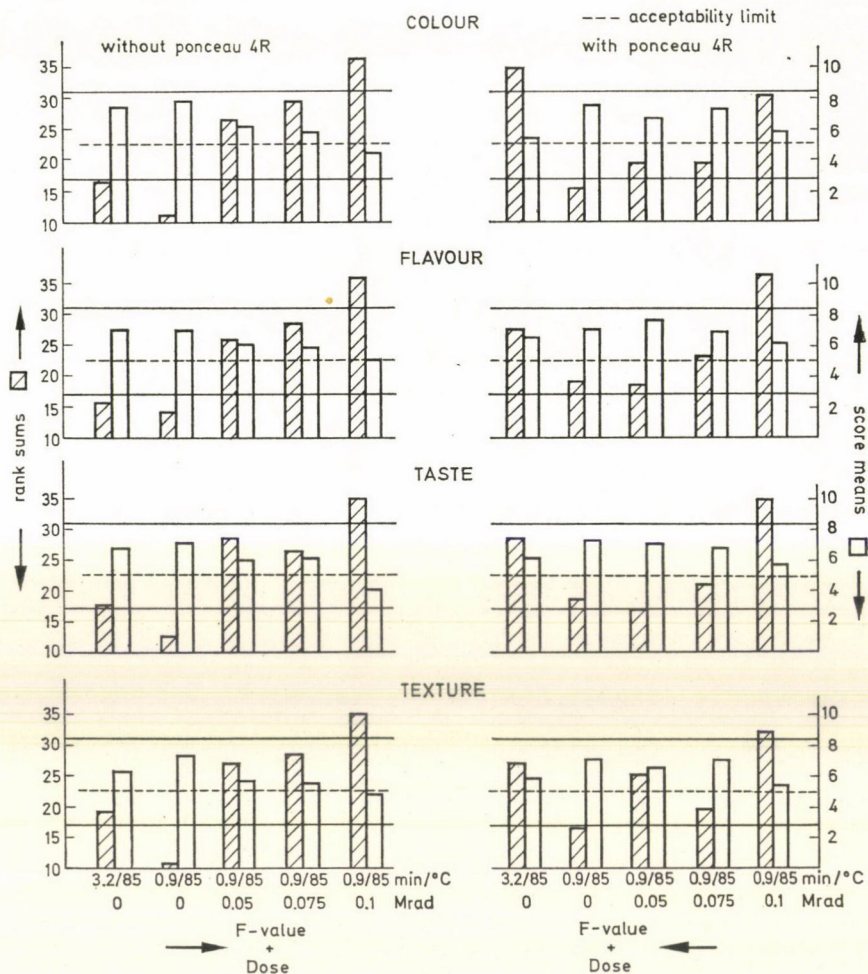


Fig. 1. Results of the sensory tests of strawberries in syrup (Senga-Sengana) with and without Ponceau 4R, 6 months stored at 20 °C. This product is preserved by means of the combination heat-radiation. The rank sums between the lowest and highest limit (17–31) do not differ to a significant degree at 95 per cent probability level. The limit of acceptability of the score means is 5. Number of panelists: 8

One hundred cans per treatment were stored during 3 years at 20, 25 and 37 °C. During this period only the unirradiated samples with an  $F$  value of 0.9 min/85 °C showed "blowing".

## 2.2. Pears in syrup

Variations in initial ripeness and discolorations render processing problematic. When pears are too ripe texture will be too weak after canning. Moreover, long pasteurization times may lead to pink discolorations (LUH *et al.*, 1960). Therefore a combined treatment might be of considerable interest.

The experiments were carried out in 0.5-l cans containing Bon Chrétien pears. The penetrometer value of the raw pears was 2.5. The control was treated at 20 min 100 °C, resulting in an  $F$  value of 10 min/85 °C. The samples were pasteurized at 16 min 100 °C ( $F$  value 7.2 min/85 °C).

Table 2 shows the test results after 12 days storage at 20 °C. The data show an increased value for the drained weight in the sequence heat-radiation; the reverse sequence does not show this clearly, probably due to a difference in the processing method (cooling after filling).

Table 2

*Effect of combined treatment upon the sensory quality of pears in syrup (Bon Chrétien) after 12 days storage at 20 °C (factory quality code)*

Heat + Irradiation	Drained weight	Colour	Flavour	Taste	Texture
$F$ value + Dose	in g	max. 25	max. 5	max. 15	max. 25
10 min/85 °C + 0 Mrad	466	19	2	12	16
7.2 min/85 °C + 0 Mrad	490	18	2	10	14
7.2 min/85 °C + 0.15 Mrad	500	22	2	11	19
7.2 min/85 °C + 0.3 Mrad	492	13	2.5	11	19
Irradiation + Heat					
Dose + $F$ value					
0 Mrad + 10 min/85 °C	507	18	3	10	20
0 Mrad + 7.2 min/85 °C	506	20	3	12	18
0.15 Mrad + 7.2 min/85 °C	492	21	3	11	20
0.3 Mrad + 7.2 min/85 °C	524	20	3	12	21

The same holds true for the colour: heat before irradiation gave a whiter colour than the control; the reverse treatment does not show this; a dose of 0.3 Mrad gives some yellow colour even. Either sequence gave an optimum result at 0.15 Mrad.

Taste improved only slightly in the sequence radiation-heat. The shorter the heat treatment the better the texture of the product: heat without radiation, however, gave a too hard product of poor consistency. The irradiated samples were rated the same or better than the control.

These experiments show that combined treatment slightly improves the quality; because of variations between the two sequences it is difficult to demonstrate a preference.



The minimum required pasteurisation time depends on the initial ripeness of the pears; fruits with a penetrometer value of 4.5, for instance, were not well-cooked at an  $F$  value of 7.2 min/85 °C.

### 2.3. *Asparagus*

The product was processed in 0.25-l cans. The control was sterilized at 22 min 115 °C with an  $F$  value of 19.3 min/115 °C. Combined treatment reduced this time to 11 min 115 °C ( $F$  value 8 min/115 °C). In order to improve colour and texture another treatment at 22 min 100 °C ( $F$  value 0.27 min/115 °C) was also investigated.

Organoleptic testing was carried out after 8 months storage at 20 °C (Fig. 2). The control was yellowish, while the combined treated asparagus remained white. Lowering the temperature to 100 °C did not improve the colour.

Doses up to 0.3 Mrad did not impair flavour or taste. The sequence heat-radiation slightly improved the samples in comparison with the control. Either sequence gave a better appreciation of the texture of the samples compared with the control. The asparagus treated at 100 °C were too hard and, consequently, judged worse. The same insufficient heat treatment showed up in the storage tests: 66% of the samples were "blown" after 6 days at 37 °C. The samples treated 11 min at 115 °C did not show this type of spoilage. One month storage of the latter in the incubator at 37 °C resulted in the following (Table 3): nearly all samples including the control still contained some meso-

Table 3  
*Bacterial growth in canned asparagus after 1 month at 37 °C*

Heat + Irradiation		Mesophilic spores		Thermophilic spores
		anaerobic	aerobic	aerobic
$F$ value + Dose	pH			
19.3 min/115 °C + 0 Mrad	5.5	$7 \times 10^2$	$5 \times 10^2$	<100
8 min/115 °C + 0.09 Mrad	5.7	<10	$7 \times 10^2$	<100
8 min/115 °C + 0.15 Mrad	5.7	<10	$1.2 \times 10^3$	<100
8 min/115 °C + 0.25 Mrad	5.7	$7.5 \times 10^3$	$5 \times 10^2$	<100
0.27 min/115 °C + 0.3 Mrad	5.0	$6 \times 10^2$	<10 <sup>2</sup>	<100
Irradiation + Heat				
Dose + $F$ value				
0.3 Mrad + 0.27 min/115 °C	4.7	$1 \times 10^4$	$1 \times 10^3$	<100

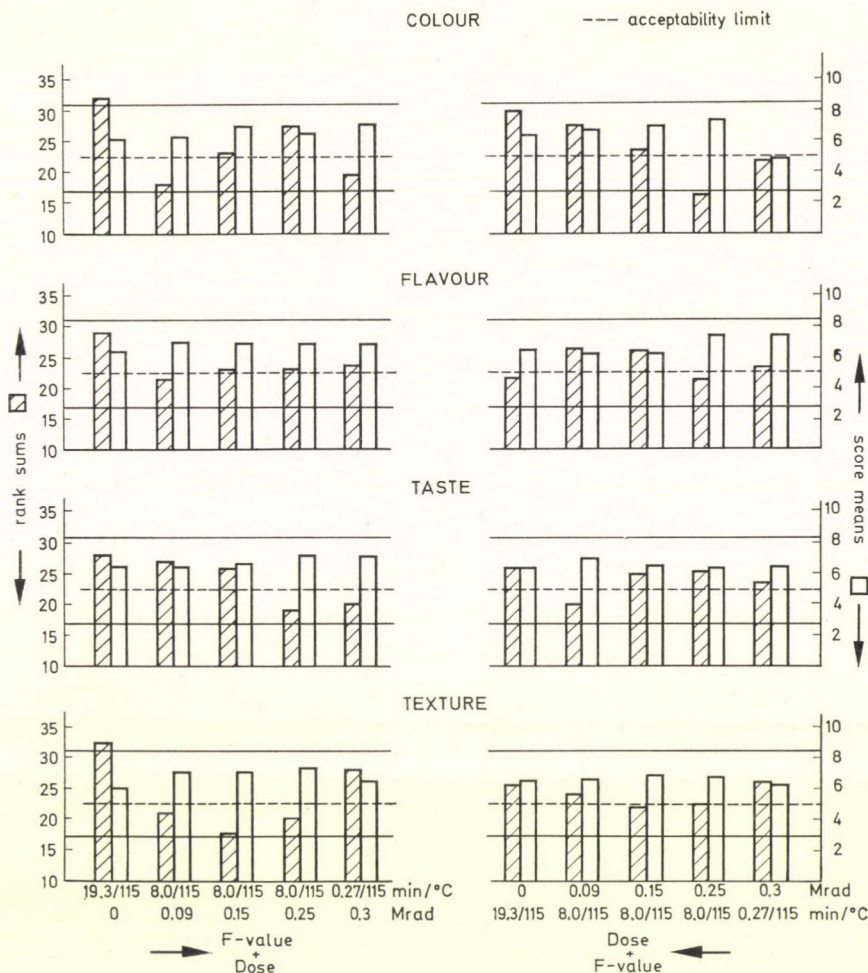


Fig. 2. Results of the sensory tests of canned asparagus stored 8 months at 20 °C. This product is preserved by means of the combination heat-radiation and the combination radiation-heat. The rank sums between the lowest and highest limit (17–31) do not differ to a significant degree at 95 per cent probability level. The limit of acceptability of the score means is 5. Number of panelists: 8

philic spores. The swelling of the samples treated at 0.3 krad + 0.27 min/115 °C correlated with a high bacterial count and with a low pH (<5).

The combined treatment slightly improved the quality of the asparagus. A minimum *F* value of 10 min/115 °C was required to achieve a well cooked product. It may be concluded that the sequence heat-radiation gave better results.



## 2.4. Spinach

The heat transfer in the spinach can is the classical example of the conduction type. In order to achieve an  $F$  value of 2.45 min/121 °C in the heart of a 1/1-l can a sterilization time of 70 min 121 °C is required. This deteriorates taste and colour; the latter by the transformation of chlorophyll into the brown pheophytine (SCHANDERL *et al.*, 1962).

The control was sterilized at 80 min 121 °C ( $F$  value 4.65 min/121 °C), for the combined treatment samples sterilization times were 20 and 40 min/121 °C, respectively. Some samples were treated at 100 °C to see if this might result in an improved colour.

Temperature measurements during treatment confirmed the bad heat transfer. The  $F$  values of the combined treatment were negligible; this holds especially true for the sequence radiation-heat where the hot filled can was cooled before radiation.

The quality testing showed an improvement of the combined treatment samples over the controls; the former were still green often directly after the heat treatment whereas the latter had turned into brown. This advantage, however, was not permanent: after three months storage at 20 °C the green turned gradually into brown. A number of the test samples kept their green colour (Table 4). These samples were either the ones with the longest heat

Table 4  
Combined treatment effect upon the sensory quality of canned spinach  
after 3 months at 20 °C

Heat + Irradiation	Vacuum	Colour	Flavour
$F$ value + Dose	cm Hg	score	score
4.65 min/121 °C + 0 Mrad	34	6.5	6.5
0.04 min/121 °C + 0.05 Mrad	34	7.0	6.0
0.04 min/121 °C + 0.1 Mrad	—	8.0	6.0
0 min/121 °C + 0.15 Mrad	6	4.0	4.0
0 min/121 °C + 0.25 Mrad	30	6.0	4.0
0 min/121 °C + 0.3 Mrad	5	2.0	4.0
Irradiation + Heat			
Dose + $F$ value			
0 Mrad + 4.65 min/121 °C	26	6.5	6.7
0.05 Mrad + 0 min/121 °C	16	4.0	4.0
0.1 Mrad + 0 min/121 °C	10	4.0	4.0
0.15 Mrad + 0 min/121 °C	2	4.0	4.5
0.25 Mrad + 0 min/121 °C	10	6.5	5.5
0.3 Mrad + 0 min/121 °C	—	7.5	6.0

treatment (40 min 121 °C) or those with the highest radiation dose (0.3 Mrad). Generally a bad colour was accompanied by an off-flavour. Moreover the vacuum tended to decrease with increasing doses (PRATT *et al.*, 1967).

Most combined-treated samples showed "blowing" after one week storage at 37 °C. This is probably related to the low *F* values. This was clearest for the sequence heat-radiation. Mesophilic spores were mostly responsible for this spoilage.

### 2.5. French beans

French beans were processed in 1/1-l cans. The control was treated at 18 min 121 °C (*F* value 17.45 min/121 °C). For the combined treatment samples the sterilization time was reduced to 10 min 121 °C (*F* value 7.35 min/121 °C). The sequence radiation-heat was treated for one minute longer because of lower initial temperature due to cooling after filling.

*F* measurements gave a value of 2.45 min/121 °C after 4 min 121 °C treatment. The beans were not fully cooked, however, after this short heat treatment. This was not even the case at an *F* value of 7.35 min/121 °C.

Organoleptic tests were carried out after 14 days and 5 months storage at 20 °C, respectively.

The former test resulted in an improved colour for the combined treatment samples (green instead of yellow-green for the control); improved flavour and taste and a better texture. The controls were starchy, weak and overcooked; the samples of the combined treatment very firm but partially not fully cooked.

The latter test demonstrated the same result as for spinach; the colour improvement was not stable. This showed best up at 0.5 Mrad. Fig. 3 shows further that the samples of the combined treatment had an inconsistent colour and the differences with the control were slight. Again taste and flavour of the combined treated samples were higher rated; the improvement was statistically not significant, however. The texture of the sequence heat-radiation was significantly better than that of the control; this was not the case with the reverse sequence. Texture differences were better distinguishable physically (tenderometer) than organoleptically (see Table 5). This table discloses further that the advantage of a better texture due to the shorter heat treatment is gradually lost at increasing irradiation dose.

Storage tests in the incubator at 20 and 37 °C did not even expose any swelling for the unirradiated samples; this can be related to the high *F* value (7.35 min/121 °C). No sequence preference could be indicated.



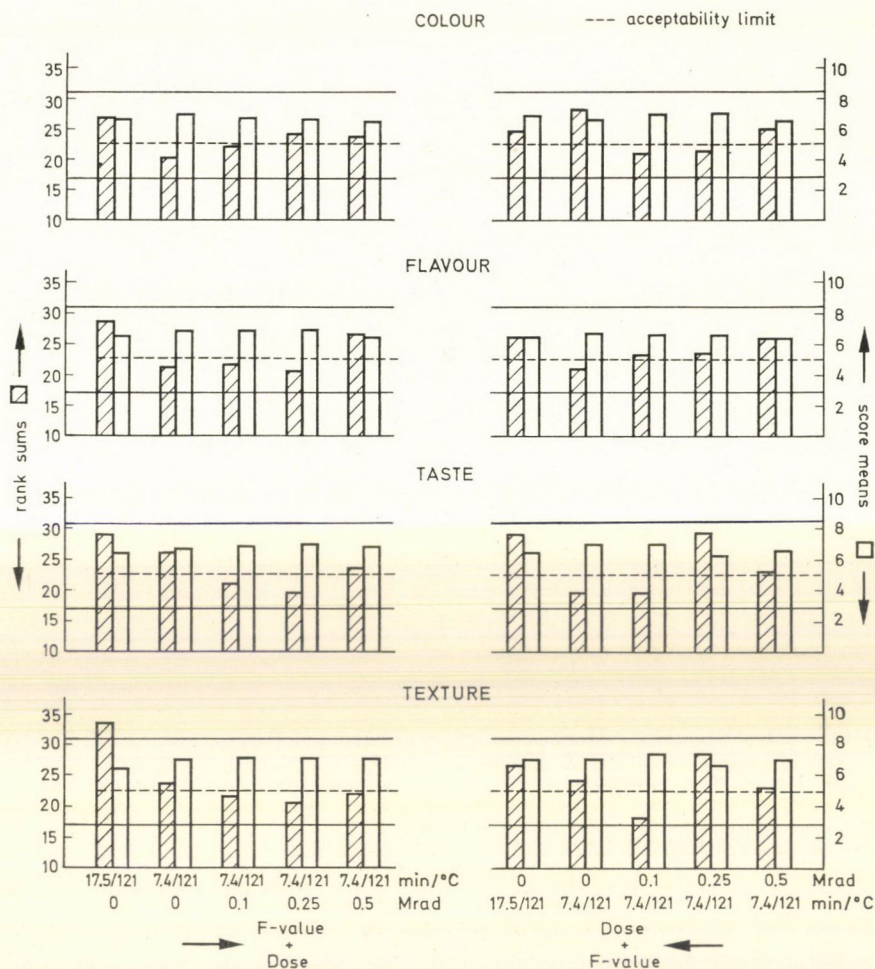


Fig. 3. Results of the sensory tests of canned French beans stored 5 months at 20 °C. This product is preserved by means of the combination heat-radiation and the combination radiation-heat. The rank sums between the lowest and highest limit (17–31) do not differ to a significant degree at 95 per cent probability level. The limit of acceptability of the score means is 5. Number of panelists: 8

## 2.6. Green peas

The experiments took place in 0.5-l cans. The peas had grade 4 (coarse) and tenderometer value of 194. The control was treated at 22 min/121° ( $F = 18$  min/121 °C). This time was reduced to 6 min ( $F = 4.9$  min/121 °C) for the sequence heat-radiation.

However, these peas having a tenderometer value of 194 were not yet fully cooked after this time. Hence it was increased to 10 min ( $F = 9.1$  min/121 °C) for the sequence radiation-heat.

Table 5

*Tenderometer values (20-kg spring) of canned French beans after 1 year at 20 °C*

Heat + Irradiation		Firmness in kg $\pm$ standard error
<i>F</i> value + Dose		
17.45 min/121 °C + 0	Mrad	10.7 $\pm$ 0.4
7.35 min/121 °C + 0	Mrad	12.8 $\pm$ 0.2
7.35 min/121 °C + 0.1	Mrad	12.3 $\pm$ 0.6
7.35 min/121 °C + 0.25	Mrad	11.3 $\pm$ 0.7
7.35 min/121 °C + 0.5	Mrad	9.8 $\pm$ 0.5
Irradiation + Heat		
Dose + <i>F</i> value		
0	Mrad + 17.45 min/121 °C	9.8 $\pm$ 0.4
0	Mrad + 7.35 min/121 °C	12.2 $\pm$ 0.6
0.1	Mrad + 7.35 min/121 °C	11.8 $\pm$ 0.7
0.25	Mrad + 7.35 min/121 °C	10.3 $\pm$ 0.2
0.5	Mrad + 7.35 min/121 °C	9.5 $\pm$ 0.5

Organoleptic testing was carried out after one and six months. At first the colour of the control was considered significantly worse than that of the combined treatment samples. The latter still retained the colour of a freshly cooked product, the former being yellowish green. The same applied after six months at 20 °C (Fig. 4). Again the differences were not so clear anymore, some samples being inconsistent in colour. This held especially true for the sequence radiation-heat.

The organoleptic tests did not confirm these findings: some testers considered the peas of yellowish green colour as normal and rated them higher than the green ones.

The flavour of the control was regarded significantly better.

Contrarily, the taste of the samples of the combined treatment was rated higher (sweeter).

The texture of the control was regarded more starchy. But again the combined treatment samples did not get a better credit: they were not yet fully cooked because of the shorter heating time. Tenderometer measurements after 1.5-year storage showed these differences clearly (Table 6). There was no blowing even after 3-year storage at 20 and 37 °C. The only incidence of thermophilic aerobic spores — as demonstrated on flat-sour-agar — was found with the unirradiated sample treated to an *F* value of 4.9 min/121 °C.



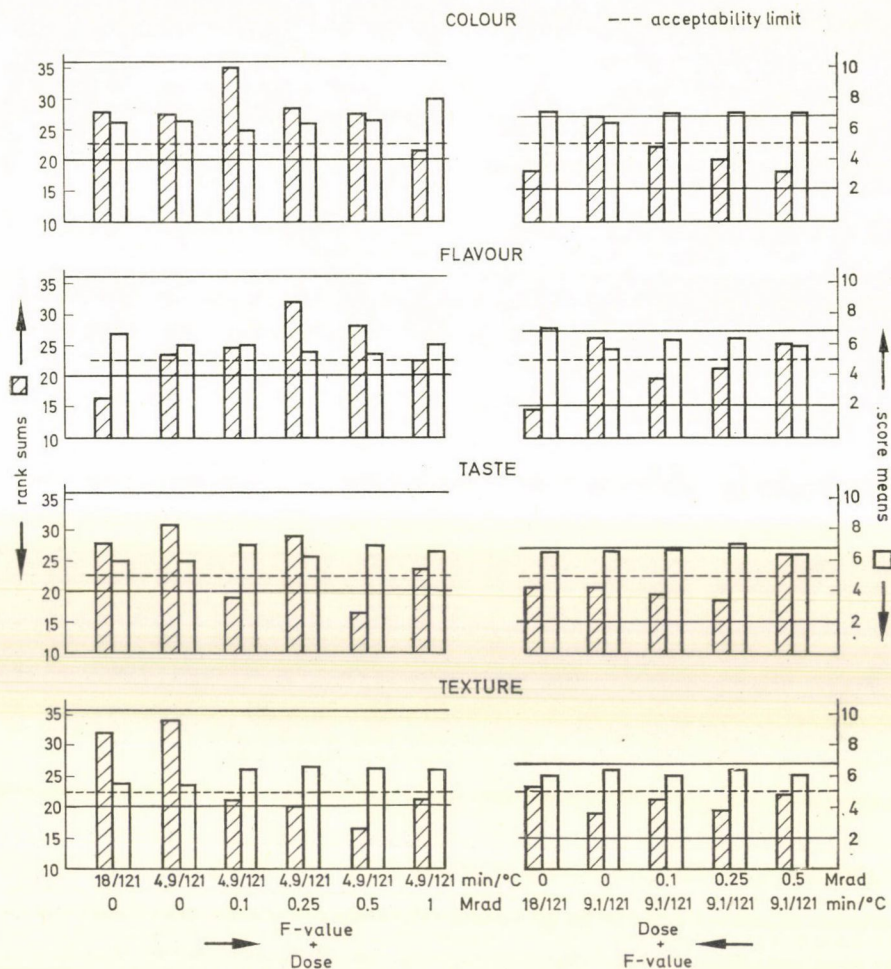


Fig. 4. Results of the sensory tests of canned peas stored 6 months at 20 °C. This product is preserved by means of the combination heat-radiation and the combination radiation-heat. The rank sums between the lowest and highest limit do not differ to a significant degree at 95 per cent probability level. These limits are for the heat-irradiation treatment (20–36) and for the irradiation-heat treatment (15–27). The limit of acceptability of the score means is 5. Number of panelists: 8

No influence of sequence on quality could be detected. Radiation doses up to 0.5 Mrad did not impair the quality in a statistically significant way.

Table 6  
*Tenderometer values (100-kg spring) of canned peas  
 after 1½ years at 20 °C*

Heat + Irradiation		Firmness (kg) $\pm$ standard error
<i>F</i> value + Dose		
18 min/121 °C + 0	Mrad	25.8 $\pm$ 1.0
4.9 min/121 °C + 0	Mrad	60.0 $\pm$ 1.5
4.9 min/121 °C + 0.1	Mrad	63.0 $\pm$ 1.7
4.9 min/121 °C + 0.25	Mrad	57.7 $\pm$ 2.3
4.9 min/121 °C + 0.5	Mrad	55.0 $\pm$ 2.8
4.9 min/121 °C + 1	Mrad	50.0 $\pm$ 1.4
Irradiation + Heat		
Dose + <i>F</i> value		
0 Mrad + 18 min/121 °C		14.0 $\pm$ 1.8
0 Mrad + 9.1 min/121 °C		26.8 $\pm$ 2.3
0.1 Mrad + 9.1 min/121 °C		24.5 $\pm$ 1.3
0.25 Mrad + 9.1 min/121 °C		25.8 $\pm$ 1.7
0.5 Mrad + 9.1 min/121 °C		27.3 $\pm$ 1.4

### 3. Conclusions

By these preliminary studies some aspects are elucidated being of interest for further research work. Table 7 gives a general view of the results.

Table 7  
*Combined treatment effect*

Product	Heat	Irradiation	Quality improvement		
	<i>F</i> value	Max. dose in Mrad	Colour	Flavour/ Taste	Texture
Strawberries	0.9 min/85 °C	<0.1	+	$\pm$	+
Pears	7.2 min/85 °C	<0.3	+	$\pm$	+
Asparagus	8.0 min/85 °C	<0.3	+	$\pm$	+
Spinach	0.04 min/121 °C	>0.3	$\pm$	$\pm$	$\pm$
French beans	10 min/121 °C	<0.3	$\pm$	$\pm$	+
Peas	9.1 min/121 °C	$\leq$ 0.5	$\pm$	$\pm$	+

+ = clear

$\pm$  = moderate



The colour of most investigated products is evidently improved by a shortened heating treatment. Unfortunately the better green colour of spinach, French beans and green peas is not stable, and deteriorates during storage. Nevertheless, it was often better than the control after one year. Doses up to 0.3 Mrad had no adverse effect on the colour except for strawberries. These faded sometimes even at 0.1 Mrad. This fading could be counteracted by the addition of an artificial coloring agent (Ponceau 4R).

The shorter heat treatment hardly affected flavour and taste if doses up to 0.3 Mrad were applied. The exception to the rule was, again, the strawberries at 0.1 Mrad.

Tenderometer measurements demonstrated a correlation between increasing irradiation dose and diminishing hardness. The required tenderness (texture) of the product restricted the range of reduction of the heat treatment. Consequently, it makes only sense to apply the combined treatment to a product when the traditional heat treatment renders it "overcooked".

The treatment sequence did not distinctly affect the quality or the microbiological spoilage. A problem at the sequence radiation-heat is that cooling after filling results in an energy dissipation which is technologically and economically unfavourable.

Finally, it is interesting to note that  $F$  values corresponding to the optimum tenderness easily satisfy the *Clostridium botulinum* concept requirements. More research on the sterilization effect of the heat-irradiation treatment, using artificially inoculated samples, is needed.

\*

Thanks are due to my colleagues for their valuable advice and particularly to Drs. R. M. ULMANN, director of The Pilot Plant for Food Irradiation, for his collaboration in the preparation of the English text.

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## COMBINED METHODS OF DECREASING WATER ACTIVITY IN FRUITS UNDER PRESERVATION\*

V. I. ROGACHEV and I. I. KISLENKO

(Received September 22, 1972)

Diffusion and osmotic methods of fruit sugar saturation, used to decrease water activity, require much time.

On the basis of qualitative and quantitative studies of the heating effect on mass exchange two new combined methods of process intensification have been suggested: 1) the combination of alternating heating and cooling procedures and 2) the combined effect of heating together with acoustic oscillations. Mass exchange observed on applying these methods is based on processes qualitatively differing from diffusion and osmosis. In the first method phase transformations of fruit cell sap water and changes of vapour pressure in fruit tissues during heating and cooling are used.

In the second one the mechanical effect of sound oscillations on fruit affecting fruit volume and removing from its surface the syrup boundary layer of weaker concentration is utilized.

The application of the new methods provide to accelerate considerably the process of fruit impregnation with sugar as compared to the standard ones practised.

The decrease in water activity to inhibit microbial activity can be achieved using different methods among which the impregnation of fruits with sugar is of practical importance (for instance, when processing candied peel and fruit preserves). Mass exchange in the fruit-sucrose solution system is based on diffusion and osmotic processes taking place at comparatively low velocity.

Their intensification by increasing temperature and concentration gradients is limited because of small temperature coefficients of diffusion and osmosis and as a result of considerably higher viscosity corresponding to higher concentration of the sucrose solution.

Besides it appeared that there is a certain limit of expediency in temperature rise. It has been found (ROGACHEV, 1951) that at a certain moment of fruit heating its mass exchange character is changing markedly. This corresponds to the points on curves of Fig. 1 where sugar penetration into the fruit is considerably slowing down and water escape from the fruit is increasing sharply. The location of the turning point of the process corresponds to the boiling point of cell sap.

\* Presented at the IUFOST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



Thus mass exchange acceleration due to temperature rise can induce mass exchange disbalance with prevalence of the moisture escape from the fruit. As a result of this fruit sugar saturation is substituted by its dehydration.

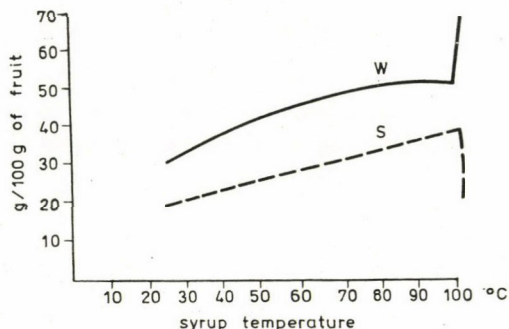


Fig. 1. Effect of temperature on the amount of water (*W*) leaving the fruit tissue and the amount of sugar (*S*) penetrating into the fruit

More efficient means of intensifying mass exchange are the combined methods of fruit treatment, which are attached to processes qualitatively differing from diffusion and osmosis. We suggested the following combined methods: 1) alternate heating and cooling procedures and 2) combined heat treatment together with acoustic oscillations.

## 1. Methods

### 1.1. Combined method of alternating heating and cooling

The combined heating and cooling method consists of heating the fruit contained in 40 to 60 per cent sucrose solution up to the boiling point and then, after keeping the fruits for a few minutes in the boiling syrup, the syrup is quickly cooled down. Heating periods followed by cooling are repeated several times (ROGACHEV, 1951).

According to this method the mass exchange is based mainly on phase transformation of fruit cell sap water and on vapour pressure changes in fruit tissues.

At a certain stage of heating cell sap water will turn into steam because the boiling point of the cell sap is lower than the boiling temperature of the sucrose solution. At this period the velocity of phase transformation is exceeding the speed of molar vapour transfer. As a result of this a considerable pressure gradient appears inside the fruit. At the period of cooling the vapour

pressure drops which results in intensive "suction" of syrup into the fruit and creates strong convection flows.

The utilization of the combined method of alternate heating and cooling provides considerable acceleration in the process of sugar impregnation yielding prepared fruits of good quality (maintenance of original fruit volume, fine texture etc.).

Intensification of mass exchange according to the described method, however, is limited by the relatively low heat conductivity of fruit and the consequent impossibility to decrease the duration of heating and cooling periods below a certain limit.

### 1.2. Combined method of heat treatment and acoustic oscillation

The second method permits to accelerate the process of mass exchange irrespectively of the thermophysical properties of fruit. This method consists of simultaneous heat treatment under 90 to 95 °C with acoustic oscillation in the range of 20 to 200 Hz (KISLENKO *et al.*, 1972).

## 2. Results and conclusions

The effect of sound pressure on mass exchange in a fruit placed in 15 per cent sucrose solution under 85 °C is shown in Fig. 2.

With acoustic oscillations applied the rate of the process increases 2 to 3 times.

Process acceleration was proved by direct observations of fruit in syrup on sound treatment and without it.

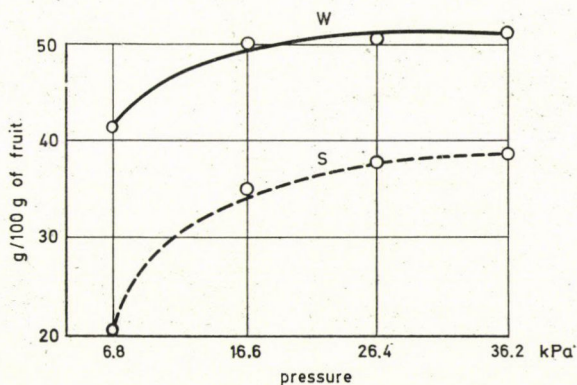


Fig. 2. Effect of sound pressure on the amount of water (*W*) leaving the fruit tissue and the amount of sugar (*S*) penetrating into the fruit



An installation for holographic research with a film camera was used (Fig. 3).

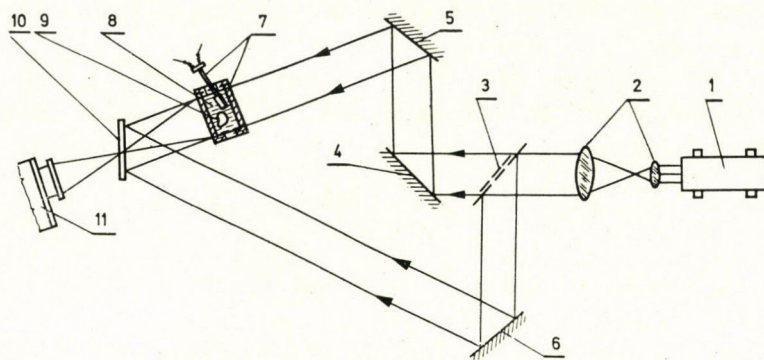
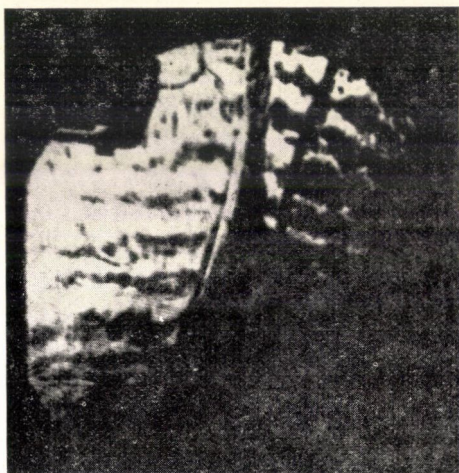


Fig. 3. Scheme of unit for holographic study of the processes in the system sugar syrup — fruit. 1 — laser; 2 — beam expander; 3 — semitranslucent specula; 4, 5 and 6 — specula; 7 — vibrator; 8 — syrup; 9 — fruit; 10 — hologramme; 11 — film camera

It was clearly shown (Fig. 4) that as a result of sound oscillations active intermixing of the medium, quick removal of the boundary layer from the division surface of the 2 phases (fruit — syrup) and the creation of intensive mass exchange in fruit are taking place.

The effect of oscillations in the medium causes periodical changes of fruit volume which favour intensive alternation of pressing out of liquid phase from the fruit and of the following fruit impregnation with the surrounding syrup. The fruit turns into a kind of pump.



a)



b)

Fig. 4. Interferogramme of the process of juice escape from fruit placed in a 40 per cent sucrose syrup: a) without acoustic oscillations: single juice flow is seen; b) as a result of acoustic oscillations a great number of juice and syrup streams arise



In addition, under the action of sound oscillations convection micro-currents of liquid phase are taking place inside the fruit and the boundary layer of the sucrose solution of weakened concentration is removed from the surface of the fruit.

Under the oscillation influence in the medium a relative rate of fruit and syrup appears, which can be characterized by the coefficient  $K_u$  (ROGACHEV & KISLENKO, 1972).

$$K_u = \frac{U_0}{U} hM,$$

where:

$U_0$  — relative velocity of fruit and liquid;

$U$  — vibrative rate of liquid;

$h$  — geometric factor (function of fruit radius and wave entering depth — the waves appearing in the medium as a result of acoustic oscillations);

$M = \frac{\rho}{\rho_0}$  — dimensionless ratio of fruit density  $\rho$  and density of medium  $\rho_0$  surrounding the fruit.

The dependence of sucrose impregnation of fruit ( $\Delta = C_t - C_i$ ) on duration ( $t$ ) of sound oscillation treatment may be expressed in terms of the exponential expression

$$\frac{d\Delta}{dt} = A.K.e^{-kt},$$

where:

$A = \frac{(1 - C_i)(C_s - C_i)}{(1 - C_s)}$  : coefficient characterizing sucrose concentration changes in fruit and syrup,

$K = (1 - C_s)f$  : coefficient characterizing the summarized velocity of fruit sugar impregnation as a result of molecular diffusion, syrup migration in pores and capillaries of fruit, convection and other factors,

$C_i$  : initial sucrose concentration in fruit,

$C_t$  : sucrose concentration in fruit during treatment,

$C_s$  : syrup concentration,

$f$  : coefficient reflecting the effect of the physical properties of fruit (number of pores per unit of fruit surface, pore and capillary forms, their sizes etc.).

It is possible to accept with a high degree of accuracy that

$$\Delta \cong A(I - e^{-kt}).$$

It is necessary to stress the suitability for this purpose of sound oscillations as compared to ultra sound because the latter oscillations having a high



fading coefficient are not effective in large capacity treatment of medium. Sound transmitters as compared to ultra sound generators are of simpler design and require less energy. It is possible to apply resonance oscillations in the low frequency range; the oscillations having a low fading coefficient help to obtain considerable acceleration in treating media of large volume.

### Literature

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## COMBINED EFFECT OF IONIZING RADIATIONS AND INFRA-RED HEATING ON FOOD PRODUCTS\*

S. YU. GELFAND and N. F. NOMEROTSKAYA

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A combined method of infra-red and ionizing radiation meat treatment has been elaborated and studied. This method guarantees *Cl. botulinum* spore suppression provided 12D is used. The lethal effect of infra-red pre-heating is equivalent to about 10D and the subsequent ionizing radiation to about 2D. With the dose of 0.6 to 0.8 Mrad necessary to obtain this effect no unfavourable chemical changes in meat have been observed and no marked changes have been found by the taste panel. After the product had been treated and following its 12 month storage total and protein nitrogen content were determined as well as the total amino acid, free amino acid, hydrogen sulfide, SH-group, lipid, volatile fatty acid level and enzyme attacking capacity of proteins. Taste panel examination has been carried out.

One of the most important use of ionizing radiation for preserving food products is the problem of pathogenic microorganism control.

To destroy vegetative cells doses under  $10^6$  rad are sufficient but much higher doses are necessary to inactivate bacterial spores. This is a serious obstacle for radiation sterilization (radappertization) of non-sour food, the microbiological standards for which are developed taking into account the high radioresistance of *Clostridium botulinum* spores.

The dose necessary to provide the safety of irradiated products during their storage at unregulated temperature was found to be 12D (similar to that used for heat sterilization). Such a dose is very high and has an unfavourable effect on the quality of irradiated products (SCHMIDT & NANK, 1960; GRECZ, 1966). Because of that various combined methods of treatment allowing to obtain minimum changes in product flavour, colour and consistency are being practised.

Thus American scientists (HARLAN *et al.*, 1967) have worked out the irradiation technology for various kinds of meat using low negative temperatures. But under such conditions of treatment higher doses (up to 5.6 Mrad) are to be applied, as the radioresistance of *Cl. botulinum* spores increases at these temperatures. In most cases meat is irradiated following heat treatment (up to 77 to 80 °C in the center of the product) necessary to complete inactivation of proteolytic enzymes.

\* Presented at the IUFoST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



We have decided to use preliminary thermal treatment as one of the main methods providing the application of low ionizing radiation doses for canning meat. To perform it we developed for meat products the method of heat treatment by infra-red rays under pressure.

### 1. Materials and methods

The scheme of the apparatus developed is given in Fig. 1.

It should be noted that the character of infra-red ray heating of products depends mainly on the generator irradiation intensity, the presence of medium in the apparatus and its parameters, the geometry of the generator and of the products as well as of their location.

To choose an appropriate generator most suitable for the task set, beef and pork spectral characteristics have been established in the range of 0.75 to 5  $\mu\text{m}$  using the spectrophotometer IKS-1. It has been found that objects studied have qualitatively similar spectra of maximum capacity in the range of 1 to 1.2  $\mu\text{m}$ . On the basis of this for a more intensive meat heating quartz lamps NIK-220-1000 of  $\lambda_{\text{max.}} = 1.0 \mu\text{m}$  were used as infra-red ray generators that correspond to the band of maximum passage. Lamps were situated on the lower and upper panels of the hermetically sealed cabinet. The roasting takes place due to the ray energy of the upper panel of the irradiation and by heat transmission from the tray heated by the lower irradiator. While heating the product part of its juice is evaporated and in the working part of the apparatus an atmosphere of overheated vapour is created. By increasing temperature pressure is increased (Fig. 2) that results in a higher boiling point of the moisture content of the product. Besides the use of overheated vapour as heat-transfer agent provides a considerable rise in the temperature of the product treated as the coefficients of heat and mass exchange between the vapour and the moist material are higher as compared to those in air. All this allows to obtain a temperature of 123 to 125  $^{\circ}\text{C}$  in the least heated spot of the product during a shorter period (of 12 to 14 min).

In the present work beef products were used as processed food (125 g) out of longissimus dorsi from carcasses following their two-day storage at the temperature of 6 to 8  $^{\circ}\text{C}$  at a slaughter house.

Prepared products were roasted in the infra-red unit. Roasted products were vacuum packed in three-layer bags (polyethylene foil—cellophane) and irradiated using the gamma-unit with a  $^{60}\text{Co}$  source at the dose rate of 530 rad/sec.

The irradiated meat products were evaluated as to their microbiological, and physicochemical properties, the nutrient content and were examined by a taste panel. The following methods have been applied. Total nitrogen content



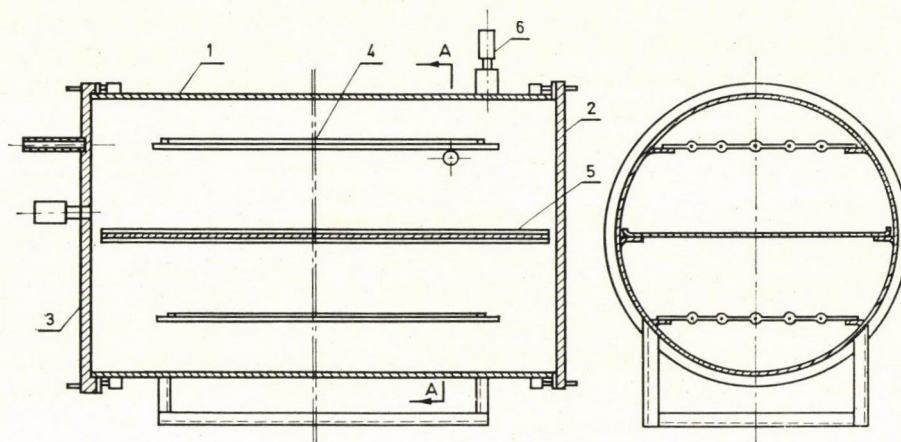


Fig. 1. Scheme of apparatus for the treatment of meat products in the infra-red field under pressure: 1. Cabinet body; 2. Cabinet door; 3. Cabinet lid; 4. Lamps; 5. Tray; 6. Manometer

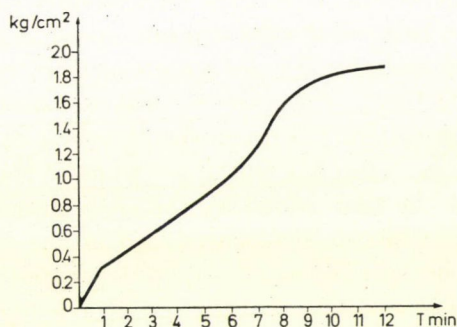


Fig. 2. Chart of pressure rise in the infra-red apparatus

was determined by the Kjeldahl method, protein nitrogen according to Burstein and Sturzer, hydrogen sulfide according to Burstein, volatile fatty acid by the distillation method. SH-groups by the sodium nitroferri cyanide reaction.

Beef lipids after their extraction according to Folch were divided into classes by thin layer chromatography using silica-gel G. Free amino acids were determined by paper chromatography and total amino acid content using the automatic Beckman analyzer. The determination of meat protein susceptible to proteolytic enzymes in vitro was carried out in the constant action apparatus for enzymatic hydrolysis and dialysis according to the method of A. A. Pokrovsky and I. D. Ertanov.



## 2. Results

### 2.1. Establishing the lethal effect of the combined treatment parameters

In our earlier work (GELFAND *et al.*, 1970) it has been shown that a taste panel found no changes in natural meat products that had been irradiated at the dose of 0.8 Mrad, thus in this case we found it expedient to use doses not exceeding 0.6 to 0.8 Mrad. Such irradiation dose can provide a  $10^2$ -fold decrease in the *Cl. botulinum* spore number (GRECZ, 1966). Consequently, the lethal effect of heat treatment applied should not be lower than 10D. As at the temperature of 121.1 °C the period D necessary for a 10-fold spore decrease is 0.3 min (ROGACHEV *et al.*, 1968) the lethal value necessary for the temperature 121.1 °C will be equal to 3.0 min.

As can be seen (Fig. 3) the practical lethal effect from overheating is approximately 10 per cent stronger.

### 2.2. Studies on the quality and nutrient value of canned meat products

The combination of heat treatment with irradiation calls forth marked and rather complicated physical and chemical changes in meat depending on heating parameters as well as on radiation effect. The main changes caused by heat treatment at temperatures over 100 °C are of hydrolytical nature and the great effect obtained is due to the long period of high temperature action (SOKOLOV, 1965).

Besides extractable nitrogenous matter destruction takes place which could be proved by the accumulation of final decomposition products, such as

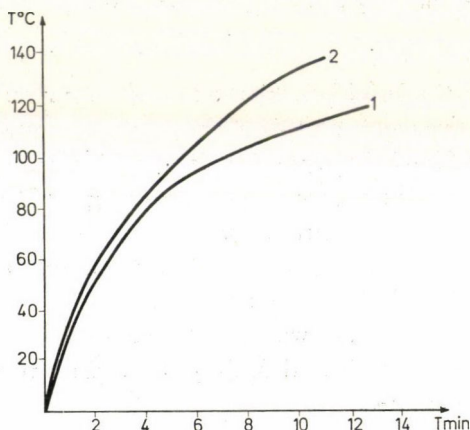


Fig. 3. Chart of temperature change in specimens roasted in the infra-red field pressure: 1. Temperature changes in the center of the specimen; 2. Temperature changes on the surface of the specimen

ammonia, hydrogen sulfide, volatile fatty acids etc. Thus in the first stage of experiments the qualitative changes in the product following its treatment in the infra-red ray apparatus and irradiation at the dose of 0.8 Mrad had to be found. Canned roasted meat prepared from a similar muscle according to the technological specification adopted was used as control. The roasted meat was vacuum packed in cans No. 9 and sterilized according to the formula  $\frac{20 - 80 - 20}{112^\circ}$ .

The data obtained from the comparative studies are given in Table 1.

Table 1  
*Quality of meat canned by different methods*

Indices	Units	Combined method		Heat sterilization
		Before irradiation	After irradiation	
Protein nitrogen	% of dry matter	11.76	11.52	11.32
Non-protein nitrogen	% of dry matter	1.28	1.52	2.06
Hydrogen sulfide	mg%	1.62	3.59	3.38
Volatile fatty acids	ml 0.1 NaOH per 100 g meat	3.05	6.82	4.35
SH-groups	$\mu\text{M}/100 \text{ mg}$	0.52	0.40	0.07

In the process of thermal treatment of meat at temperatures over 100 °C products enzymatically resistant to the interaction of proteins and peptides with non-protein components of foodstuffs might be formed. Hardly soluble protein modification non-typical for native proteins containing peptide bonds with lysine  $\epsilon$ -amino groups and free carboxyl groups of dicarbonic amino acids as well as cyclisation at certain points of the polypeptide chain are possible. As a result of possible changes the speed of meat protein digestion and utilization by the organism might be decreased (MINDLINA, 1950).

Besides the irradiation might have a marked effect on protein properties, included its response to enzymatic digestion.

This was the basis for carrying out comparative experiments as to the ability of proteolytic enzymes to attack canned meat products. The results of these experiments are given in Table 2.

From the data obtained it might be concluded that the bulk of hydrolysis products (70 to 80 per cent) is found to accumulate after the first hour of incubation. Despite of certain differences in the speed of decomposed products accumulation of their final amount was found to be practically similar in all the specimens.

At the next stage of work studies into the quality, and nutrient value of meat products canned by the combined method in the process of long-term (12 months) storage have been carried out. The studies on the lipids by thin-



Table 2

*Accumulation of hydrolysis products during the digestion of roasted meat proteins by the pepsin-trypsin method (enzyme-substrate ratio 1:10; pepsin — pH 1.2; trypsin — pH 8.2)*

Hydrolysis period, hours	Enzyme	Specimens studied		
		Meat roasted in the infra-red ray unit		Canned "roasted meat"
		Before irradiation	After irradiation	
		Hydrolysis products in mg of tyrosin nitrogen per 100 mg nitrogen		
1	pepsin	36.0	36.1	42.3
2	pepsin	37.8	36.7	43.8
3	pepsin	40.4	37.4	45.0
4	trypsin	48.6	46.0	51.7
5	trypsin	50.2	47.2	51.7
6	trypsin	53.5	50.6	52.6

Table 3

*Amino acid content of meat products canned using the combined method*

Amino acids	Content in specimens, % related to protein						
	Raw meat	Meat roasted in the infra-red apparatus irradiated at 0.6 Mrad					
		Non-irradiated	Irradiated	1 month	3 months	6 months	12 months
Lysine	7.13	7.41	6.15	7.17	7.56	6.03	7.81
Histidine	2.57	2.78	1.75	3.04	2.40	1.88	2.81
Arginine	5.08	4.70	4.79	5.03	5.39	4.50	5.97
Aspartic acid	6.32	8.51	7.17	8.95	8.58	6.69	7.92
Threonine	2.90	3.70	3.40	3.50	3.86	3.05	3.99
Serine	2.49	3.16	3.15	3.36	3.58	2.72	3.43
Glutamic acid	8.93	14.04	12.08	13.31	14.08	9.70	14.09
Proline	3.44	3.39	3.47	4.03	3.19	3.06	4.30
Glycine	3.74	4.17	3.68	4.18	3.80	4.01	3.87
Alanine	4.09	5.15	4.61	5.34	5.12	4.64	5.06
Valine	3.41	2.93	2.77	3.23	3.13	2.42	2.56
Methionine	1.30	2.03	1.83	0.73	—	1.18	0.90
Isoleucine	3.40	2.84	2.68	2.97	2.97	2.31	3.91
Leucine	5.62	6.35	5.91	6.45	6.70	5.27	6.64
Tyrosin	2.09	2.70	2.30	2.86	3.02	2.07	2.77
Phenylalanine	2.79	3.15	2.65	3.29	3.29	2.69	3.54

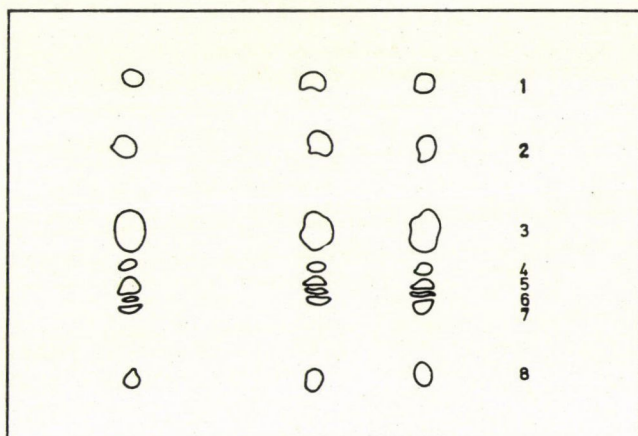


Fig. 4. Roasted meat lipid chromatogram: 1. hydrocarbons; 2. triglycerides; 3. sterol ethers; 4. free fatty acids; 5. diglycerides; 6. cholesterol; 7. monoglycerides; 8. phospholipids. Starting line: at position 8 (at the bottom). Samples (from left to right): raw meat; traditionally roasted sample; infra-red roasted meat

layer chromatography (Fig. 4) revealed the presence of 8 classes of compounds: hydrocarbons, sterol ethers, triglycerides, free fatty acids, diglycerides, cholesterol, monoglycerides and phospholipids.

Among the changes noted, following the 12 months of storage, some decrease in the triglyceride fraction and a higher free fatty acid level could be noted.

The data obtained when studying the amino acid content of meat products during cooking and storage are given in Table 3.

### 3. Conclusions

The combined process of thermal treatment in the infra-red ray apparatus and irradiation provides the necessary safety level (12D).

One of the advantages of the method discussed is the possibility to avoid supplementary fat addition during meat roasting. It is because of this fact that the thermal effect on fat calls forth its partial oxidation. The effect of ionizing radiation results in the activation of these processes and the products of fat oxidizing deterioration have an unfavourable effect on meat product quality.

The results of determining various forms of nitrogen testify to the fact that considerably less (about 30 per cent) of non-protein nitrogen was found to accumulate in infra-red heated and irradiated meat. A marked difference in sulfhydryl group content reveals considerable denaturing changes in the protein system in canned products following autoclave sterilization. Comparing



the importance of hydrogen sulfide in both groups of canned products it is not difficult to note the specific effect of ionizing radiation connected with radio-lability of sulfur containing amino acids. At the same time it should be noted that practically similar amounts of  $H_2S$  formed during canning using different methods once again prove that hydrogen sulfide is not a main component causing canned meat odour. This was established by comparative evaluation of meat products by a taste panel. Thus meat treated by a combined method had a flavour and colour characteristic of the roasted product. In these specimens no specific autoclaved meat odour was found.

The combined meat treatment process does not cause any marked changes in amino acids except the sulfur-containing ones.

Thus the experiments carried out showed that the quality of the roasted product remained at a rather high level during the whole shelf-life.

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## THE EFFECT OF COMBINED HEAT AND IRRADIATION TREATMENT ON THE ISOELECTRIC AND SIZE PROPERTIES OF HORSE-RADISH PEROXIDASE\*

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A study of the effects of heat and irradiation on peroxidase was undertaken with the aim of gaining a better understanding of the molecular processes which proceed during inactivation in foodstuffs. Most of the experiments were carried out with thin-layer isoelectric focusing in granular gels and thin-layer gel filtration, by which both the charge and size properties of enzymes can be studied. Whereas high amounts of enzymatically active aggregates were observed on irradiation, only trace amounts were found on heating. The isoelectric patterns of the remaining monomers were markedly modified by both treatments, with some distinct differences. Of the combined treatments, the sequence irradiation followed by heating gave a more efficient inactivation than the reversed sequence. The radiation-induced aggregates and the radiation-modified monomers possessed a much higher heat-lability than the untreated peroxidase. For the sequence heating followed by irradiation the modification of the monomers by the heat treatment resulted in a larger percentage of enzymatically active aggregates.

Radiation preservation of food has been successfully applied to a number of products to obtain microbiologically stable foodstuffs. Due to the high radio-resistance of enzymes "*in situ*", the endogenous enzyme systems remain active in irradiated food, and may cause undesirable changes in the organoleptic properties of the irradiated food during storage (IAEA, 1969). The heat lability of most enzymes suggests a combined heat and irradiation treatment. A combination of a comparatively light heat treatment with a feasible radiation dose appears to be a promising approach not only for controlling residual enzyme activity but also for controlling pathogenic and food spoiling microorganisms (DIEHL, 1971). Synergistic effects could lead to a better quality as compared to an only heat-treated product.

Little is known on the mechanism of enzyme inactivation by a combined heat and irradiation treatment. In most reported studies enzyme inactivation has been investigated by determination of total enzyme activity after treatment, providing only a limited insight into the molecular processes leading to enzyme inactivation. The fact that most enzymes in food occur as mixtures of isoenzymes which could differ in their inactivation characteristics, was

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mostly ignored. The residual enzyme activity observed after treatment could thus result from resistant isoenzymes.

Due to the complexity of food it is very difficult to investigate the mechanism of enzyme inactivation "*in situ*". Studies with model systems could be anticipated to provide a better insight into the molecular events of enzyme inactivation. In view of the technological importance of peroxidase in food processing and food analysis, we have chosen this highly heat-resistant enzyme as the model substance (DELINCÉE *et al.*, 1971a, b).

Commercial preparations of horse-radish peroxidase constitute a complex mixture of isoenzymes (DELINCÉE & RADOLA, 1970). Individual isoenzymes can be isolated in high purity from this mixture by a combination of different fractionation procedures (DELINCÉE & RADOLA, 1971, and in preparation). The inactivation of individual isoenzymes can thus be studied in addition to the whole isoenzyme system as it occurs in foodstuffs.

The radiation- and/or heat-induced changes of the enzyme were mainly investigated with two relatively simple methods: thin-layer isoelectric focusing in granular gels and thin-layer gel filtration. These techniques provide an information on two important physico-chemical parameters of enzymes: namely the charge properties and the size properties.

## 1. Materials and methods

### 1.1. Material

Horse-radish peroxidase with an absorbance ratio  $A_{403\text{nm}}/A_{278\text{nm}}$  of 0.6 was purchased from Boehringer (Mannheim, FRG).

### 1.2. Irradiation and heat treatment

A monomeric material was obtained by column gel filtration on Sephadex G-200, by which the absorbance ratio increased to about 1.5 without changes in the isoelectric pattern. The peroxidase was dissolved in a 0.01 *M* phosphate buffer (pH 7.2) so as to contain approx. 1% protein as determined by absorbance measurements at 278 nm. The solution was deaerated, flushed with argon and sealed in ampoules. Irradiation was carried out at 0 °C in a  $^{60}\text{Co}$  Gammacell 220 (dose rate about 1 Mrad/h). The heating was performed in a temperature-controlled ( $\pm 0.1$  °C) waterbath for the desired time (5, 10, 20 and 40 min) at 90 °C. Immediately after heating the ampoules were immersed in ice-water.



### 1.3. Peroxidase assay

Peroxidase was assayed by a slightly modified procedure of CHANCE and MAEHLY (1955). The increase in absorbance by the peroxidation of guaiacol was recorded with a PMQ II spectrophotometer (Zeiss, Oberkochen, FRG) connected with a Servogor recorder (Goerz, Wien, Austria). Initially, the reaction was linear in the interval  $0.1 < \Delta E/\text{min} < 1.5$ .

### 1.4. Thin-layer isoelectric focusing

Thin-layer isoelectric focusing was carried out as described previously (RADOLA, 1969, 1972; DELINCÉE & RADOLA, 1970). Briefly, thin-layer isoelectric focusing was performed on 20 cm × 20 cm glass plates coated with a 0.75 mm layer of a suspension of a granular gel (Sephadex G-75 Superfine, Pharmacia, Uppsala, Sweden), containing 1% of carrier ampholytes (LKB, Bromma, Sweden). After gentle drying to remove about 20% of water, the final thickness of the layer was 0.6 mm. Focusing was carried out in the Desaga double-chamber (Desaga, Heidelberg, FRG) with water at 4–10 °C circulated through the cooling block. When a potential of direct current is applied to the layer, a stable pH-gradient is built up by the carrier ampholytes, which are collected at their isoelectric points. Proteins migrate in this pH-gradient until they reach the region corresponding to their isoelectric point, and they will collect and will be focused at this particular pH.

### 1.5. Thin-layer gel filtration

Thin-layer gel filtration was carried out as described previously (RADOLA, 1968).

### 1.6. Print technique

Both in thin-layer isoelectric focusing and in thin-layer gel filtration the print technique was used in order to locate the separated components. A chromatographic paper is rolled onto the gel layer, and the liquid phase with the separated proteins is soaked up. The resulting print can be stained for proteins with Amido Black 10 B or Coomassie Brilliant Blue G-250. For enzyme detection a buffered paper with an optimal pH is impregnated with the corresponding substrates (DELINCÉE & RADOLA, 1972). For peroxidase a citrate-phosphate buffer, pH 5.0 was used. Urea-peroxide was employed as primary, and o-toluidine as secondary substrate (DELINCÉE & RADOLA, 1971, 1972). The stained print can be used for densitometric evaluation, and kept conveniently for future reference. An attractive feature of the thin-layer techniques as



compared to the column techniques is the possibility to separate simultaneously several samples on a single plate, *e.g.* untreated and treated samples. The effect of a treatment can thus be recognized easily.

## 2. Results

Thin-layer isoelectric focusing of commercial horse-radish peroxidase with an absorbance ratio of 0.6 revealed about 20 distinct isoenzymes with isoelectric points distributed over a wide pH range (DELINCÉE & RADOLA, 1970). No size differences between the isoenzymes were observed by gel filtration. A molecular weight of about 40 000 was found in agreement with other reports (PAUL, 1963).

### 2.1 Irradiation

On irradiation of this complex isoenzyme mixture the isoelectric pattern was extensively modified (Fig. 1). In the pH 4–6 range multiple enzyme zones appeared which in the unirradiated control were absent or apparent only in much smaller amounts. Quantitative changes in the activity distribution were observed; the amount of the most acidic and basic isoenzymes decreased. The difference in inactivation behaviour of the individual isoenzymes is obvious.

Thin-layer gel filtration of the irradiated peroxidase revealed enzymatically active aggregates (Fig. 2a, b). These results are in agreement with previous findings on other enzymes (HASKILL & HUNT, 1967; JUNG & SCHÜSSLER,

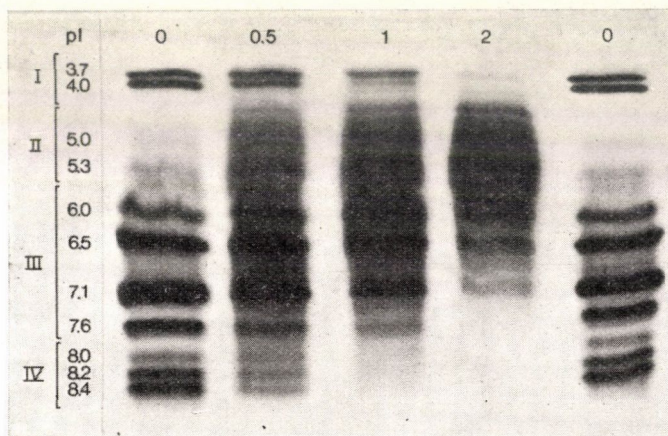


Fig. 1. Thin-layer isoelectric focusing of irradiated horse-radish peroxidase (1% protein) in pH 3–10 ampholytes. Doses in Mrad. Anode at the top. To the left the isoelectric points (see DELINCÉE & RADOLA, 1970). Enzyme detection with urea-peroxide and o-toluidine. Equal sample volumes were applied



1966; STEVENS *et al.*, 1969). With increasing dose the relative amount of the aggregated active enzyme increased. Radiation-induced aggregation of horse-radish peroxidase probably proceeds through an enzymatically active dimer, indicated by the appearance of a component with a molecular weight of about 100 000. The aggregation depended on concentration. On irradiation resulting in equal inactivation, we found less aggregation in more diluted solutions.

The monomer, dimer and higher aggregates of irradiated peroxidase were isolated by preparative gel filtration. Thin-layer isoelectric focusing of the isolated fractions revealed striking differences in the isoelectric patterns

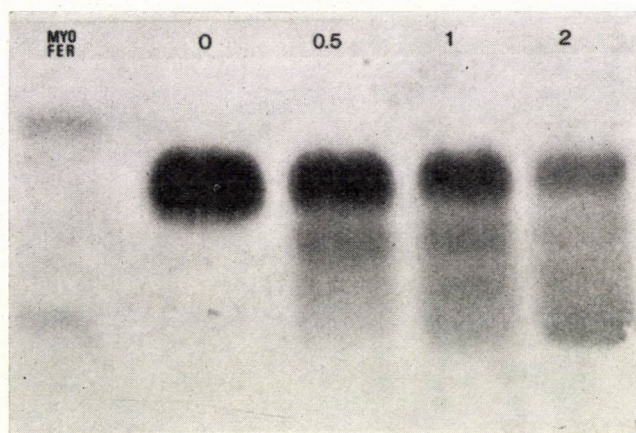


Fig. 2a. Thin-layer gel filtration of irradiated horse-radish peroxidase (1% protein) on Sephadex G-200 Superfine. Doses in Mrad. To the left the reference-proteins myoglobin and ferritin. The starting line is at the top. Enzyme staining. Equal sample volumes

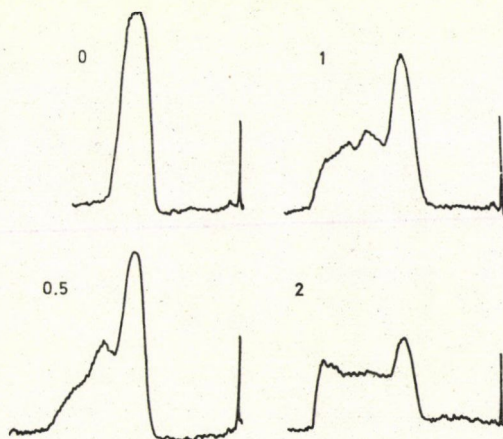


Fig. 2b. Densitograms (reflectance) of irradiated horse-radish peroxidase, separated by thin-layer gel filtration (see Fig. 2a). The peak to the right corresponds to the starting line



(Fig. 3). The dimer and the higher aggregates exhibited a very similar pattern with little resemblance to the pattern of the control. They consisted mainly of multiple enzyme zones with isoelectric points in the pH 4–6 range, which accumulated with increasing dose. The radiation-induced changes in the monomers (Fig. 4) resembled those of the unfractionated peroxidase. The most basic and acidic isoenzymes disappeared, and increasing amounts of the pH 4–6 components were produced. The changes of the charge properties of the monomer as revealed by thin-layer isoelectric focusing clearly indicate a sequence of radiolytic degradations. Radiolysis of basic amino acids (LIEBSTER & KOPOL-

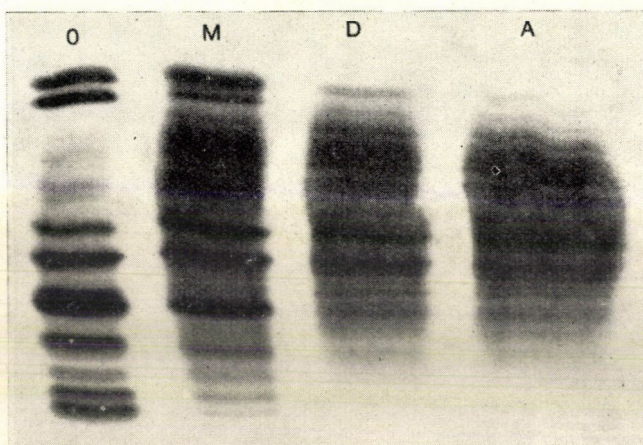


Fig. 3. Thin-layer isoelectric focusing (pH 3–10 ampholytes) of fractions of horse-radish peroxidase irradiated with 1 Mrad. Anode at the top. O: unirradiated control; M: monomer; D: dimer; A: higher aggregates; M, D, A were isolated by preparative gel filtration of the irradiated peroxidase. Enzyme staining. Amounts of equal activity were applied

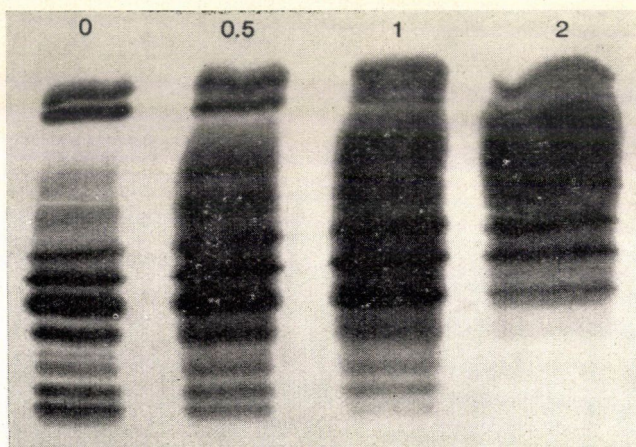


Fig. 4. Thin-layer isoelectric focusing (pH 3–10 ampholytes) of the monomers, isolated by preparative gel filtration of horse-radish peroxidase irradiated with increasing doses (Mrad). Anode at the top. Enzyme staining. Amounts of equal activity were applied



DOVA, 1964; OHTSUKI *et al.*, 1970), splitting of labile amide groups (MEE & ADELSTEIN, 1967; ROBINSON *et al.*, 1970; SATTERLEE *et al.*, 1971) and deamination (SIMIC & HAYON, 1971) could all contribute to the shift to higher acidity. The decrease in the content of the most acidic isoenzymes indicates that other mechanisms are also involved. Splitting off of acetic and propionic acid, as already found on irradiation of other proteins (FRIEDBERG & RIESZ, 1970), could well account for these changes.

Experiments with isolated individual isoenzymes (an acidic, a basic and two neutral isoenzymes) basically confirmed the results with the whole iso-

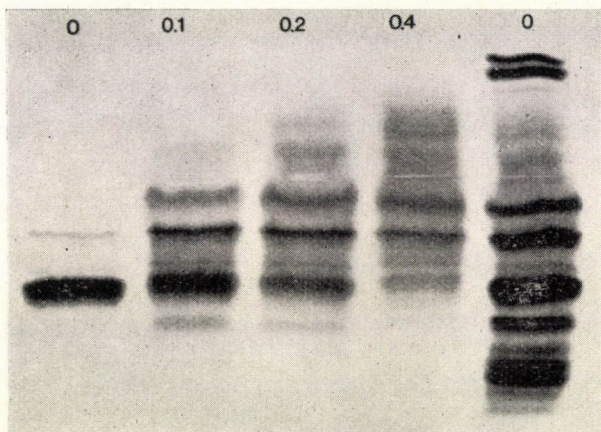


Fig. 5. Thin-layer isoelectric focusing (pH 3–10 ampholytes) of the pI 7.1 isoenzyme of horse-radish peroxidase. The isolated isoenzyme (0.1% protein) was irradiated with 0, 0.1, 0.2 and 0.4 Mrad. To the right the unfractionated enzyme. Anode at the top. Enzyme staining. Amounts of equal activity were applied

enzyme mixture. On irradiation of the basic and neutral isoenzymes a shift to higher acidity was observed by thin-layer isoelectric focusing (Fig. 5). The acidic isoenzyme was converted to components with higher isoelectric points. With increasing dose most of the activity accumulated in the pH 4–6 range for all isoenzymes. Also for the individual isoenzymes thin-layer gel filtration has proved that concentration-dependent aggregation was one of the mechanisms of radiation-induced inactivation.

## 2.2. Heat

The results obtained on heat treatment of horse-radish peroxidase differed distinctly from those obtained on irradiation. Thin-layer isoelectric focusing revealed that the basic isoenzymes were preferentially inactivated. With increasing heating time the relative amount of the acidic isoenzymes increased (Fig. 6). The isoelectric patterns showed quantitative changes with a shift to



enzymatically active components with lower isoelectric points. In contrast to irradiation, only a small amount of new enzymatically active components were formed on heating.

By thin-layer gel filtration of the heat-treated peroxidase we found very large aggregates already at low degrees of enzyme inactivation. These aggregates possessed, however, only traces of enzyme activity (Fig. 7). This is again

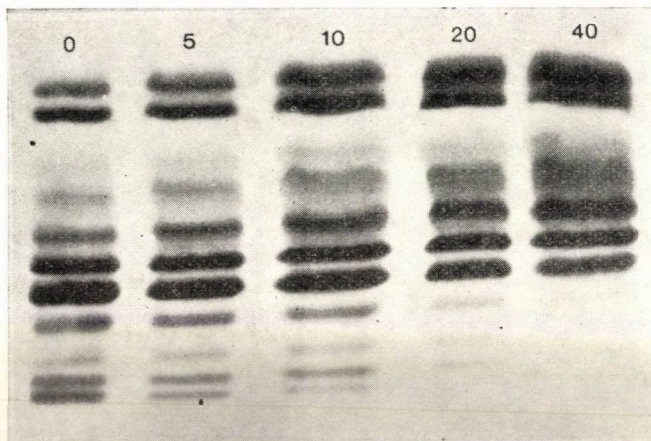


Fig. 6. Thin-layer isoelectric focusing (pH 3—10 ampholytes) of heat-treated horse-radish peroxidase (1% protein). Heating time at 90 °C (min). Anode at the top. Enzyme staining. Amounts of equal activity were applied

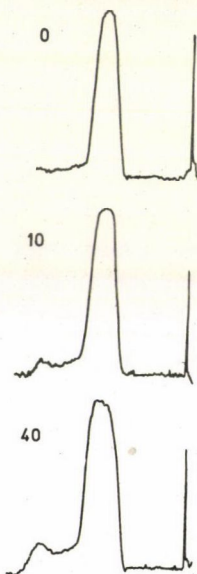


Fig. 7. Densitograms (reflectance) of heat-treated horse-radish peroxidase (1% protein), separated by thin-layer gel filtration of Sephadex G-200 Superfine. Heating time at 90 °C (min). The peak to the right corresponds to the starting line. Enzyme staining. Amounts of equal activity were applied

at variance with the radiation inactivation of peroxidase, where aggregation apparently proceeds through a dimer, trimer etc. to the larger aggregates, and where the activity of the aggregates comprises a relatively high part of the total enzyme activity.

The isoelectric patterns of the monomers after heating suggest a stepwise degradation by which the content of basic isoenzymes is reduced. Splitting of

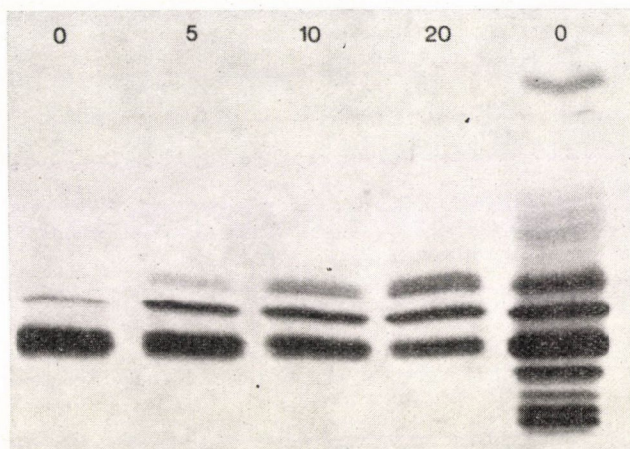


Fig. 8. Thin-layer isoelectric focusing (pH 3–10 ampholytes) of the pI 7.1 isoenzyme of horse-radish peroxidase. The isolated isoenzyme (0.1% protein) was heated for 0, 5, 10, and 20 min at 90 °C. To the right the unfractionated enzyme. Anode at the top. Enzyme staining. Amounts of equal activity were applied

labile amide groups or deamination could offer an explanation for these effects, as already proposed for irradiation. Contrary to radiolytic degradation of the monomeric enzyme, the multiplicity of induced enzyme zones, mainly in the pH range 4–6, was not observed for the heat-degraded monomer. The most acidic isoenzymes did not disappear in the isoelectric pattern of the heat-treated enzyme, only a distribution to more acidity was observed.

With the isolated individual isoenzymes these results of heating have been basically confirmed (Fig. 8). Evidently, the molecular processes leading to the inactivation of peroxidase by heat or by irradiation are different.

### 2.3. *The combined irradiation and heat treatment*

The sequence irradiation followed by heating was compared with the reversed sequence: heating followed by irradiation. A typical experiment with the whole isoenzyme mixture is shown in Table 1. The samples were treated for 5 min at 90 °C immediately before or after the radiation treatment (0.5, 1 and 2 Mrad).



Table 1

*The effect of a combined irradiation and heat treatment on horse-radish peroxidase*

Protein: 1%

Treatment	min at 90 °C	Dose (Mrad)			
		0	0.5	1	2
		Residual activity in %			
1. irradiation	0	100	70	46	21
2. (irradiation + heating) calc.	5	57	40	26	12
3. heating + irradiation	5		40	24	13
4. irradiation + heating	5		11	4	0.5
		Synergistic factor (F)			
5. $\frac{(\text{irradiation} + \text{heating}) \text{ calc.}}{\text{heating} + \text{irradiation}}$			1.0	1.1	0.9
6. $\frac{(\text{irradiation} + \text{heating}) \text{ calc.}}{\text{irradiation} + \text{heating}}$			3.6	6.5	24

The first horizontal line shows the values of residual activity after irradiation only. The values of the second line have been calculated assuming an additive action of heat and irradiation. The values of the third and fourth line have been found experimentally for the sequence heating and irradiation and for the reversed sequence. It is obvious that irradiation followed by heating is much more efficient in inactivation than the reversed sequence. The synergistic factor  $F$ , defined as the ratio of the residual activity assuming an additive action to the residual activity of the experimental values, indicates that the synergistic effect of the sequence irradiation and heating increases with increasing dose of radiation.

The isoelectric patterns of peroxidase following a combined treatment were similar to those obtained after irradiation alone. Also after a combined treatment the basic isoenzymes were preferentially inactivated, for the near-neutral isoenzymes a shift to more acidity was noted, and multiple new enzymatically active components were observed, mainly in the pH 4–6 range.

For the sequence heating followed by irradiation (Fig. 9) the accumulation of zones in the pH 4–6 range was even more pronounced than for the irradiation treatment alone. With prolonged heating before irradiation the amount of basic zones was further reduced, and a still higher accumulation in the pH 4–6 range was observed.

For the sequence irradiation followed by heating (Fig. 10) the rapid inactivation was clearly demonstrated. Only traces of activity could be observed in samples irradiated with 2 Mrad and then heated for 5 min at 90 °C. In contrast to the reversed sequence relatively small amounts of activity were found in the pH 4–6 range. Based on previous findings on irradiation applied as a



single treatment, the results of isoelectric focusing after application of a combined treatment indicate that for the sequence heating followed by irradiation enzymatically active aggregates accumulate, whereas for the reversed sequence the radiation-induced aggregates are inactivated by the subsequent heat treatment.

The indirect evidence provided by isoelectric focusing was substantiated by thin-layer gel filtration. For the sequence heating followed by irradiation (Fig. 11) the modification of the monomers by the heat treatment resulted in

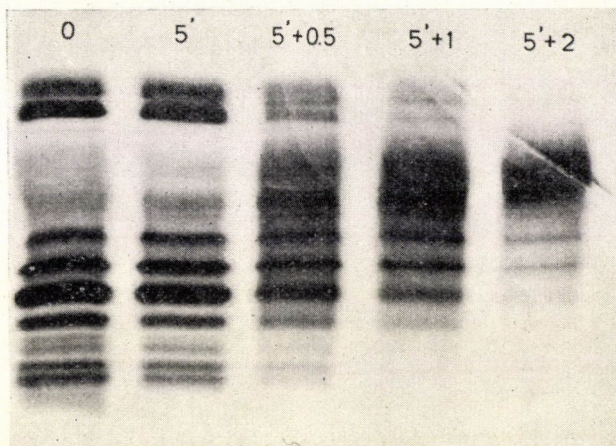


Fig. 9. Thin-layer isoelectric focusing (pH 3–10 ampholytes) of horse-radish peroxidase (1% protein) after a combined irradiation and heat treatment. Sequence: heating (5 min at 90 °C) followed by irradiation (0, 0.5, 1, and 2 Mrad). Anode at the top. Enzyme staining. Equal sample volumes were applied

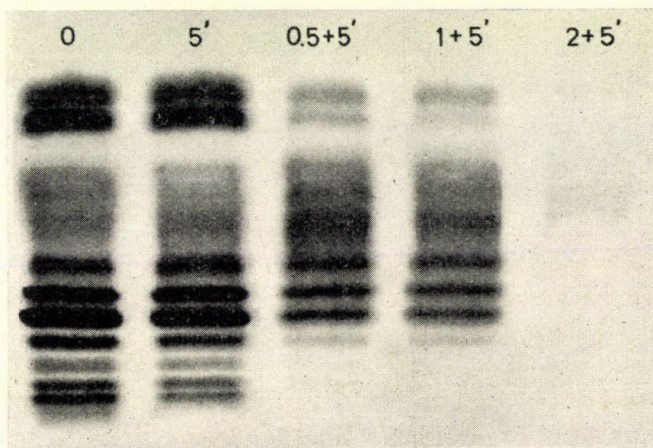


Fig. 10. Thin-layer isoelectric focusing (pH 3–10 ampholytes) of horse-radish peroxidase (1% protein) after a combined irradiation and heat treatment. Sequence: irradiation (0, 0.5, 1, and 2 Mrad) followed by heating (5 min at 90 °C). Anode at the top. Enzyme staining. Equal sample volumes were applied



a larger percentage of radiation-induced enzymatically active aggregates. The ratio of enzymatically active aggregates to the active monomer was approx. 1 : 1 after irradiation with 2 Mrad applied as a single treatment (Fig. 2a), and about 1 : 3 after pre-heating for 5 min at 90 °C (Fig. 11). Prolonged heating led to a further increase of the amount of enzymatically active aggregates — in good agreement with the enhanced modification of the monomers on heating as shown by their isoelectric patterns.

For the sequence irradiation followed by heating (Fig. 12) the amount of enzymatically active aggregates was decidedly lower than that for irradiation

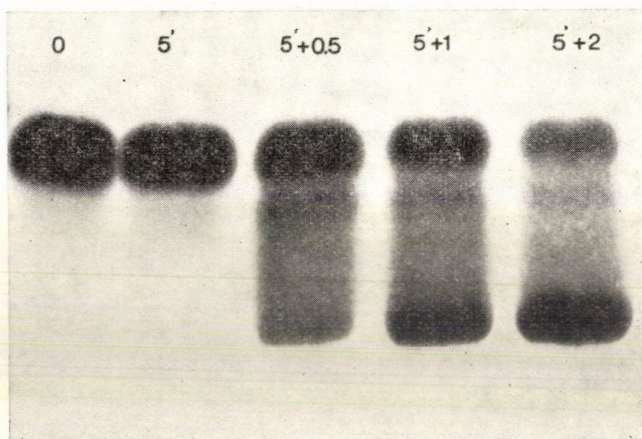


Fig. 11. Thin-layer gel filtration (Sephadex G-200 Superfine) of horse-radish peroxidase (1% protein) after a combined irradiation and heat treatment. Sequence: heating (5 min at 90 °C) followed by irradiation (0, 0.5, 1, and 2 Mrad). The starting line is at the top. Enzyme staining. Equal sample volumes were applied

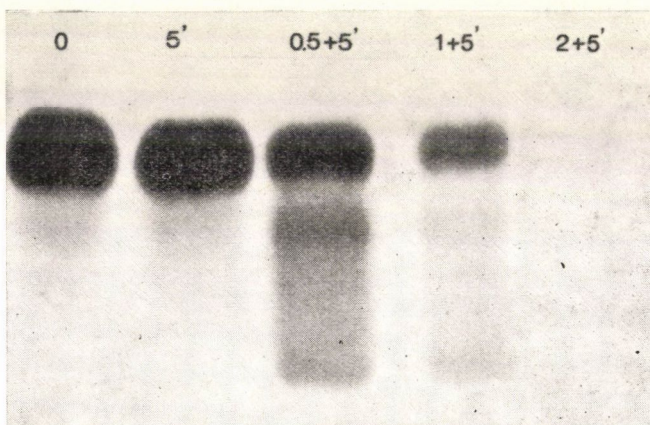


Fig. 12. Thin-layer gel filtration (Sephadex G-200 Superfine) of horse-radish peroxidase (1% protein) after a combined irradiation and heat treatment. Sequence: irradiation (0, 0.5, 1, and 2 Mrad) followed by heating (5 min at 90 °C). The starting line is at the top. Enzyme staining. Equal sample volumes were applied



alone. However, when the gel filtration print was stained for protein with Amido Black, rather than for peroxidase activity, large amounts of inactive aggregates were observed. These results indicate that the radiation-induced aggregates are preferentially inactivated by heating after irradiation.

An increased heat-lability was observed for all fractions isolated from irradiated peroxidase (Table 2). In these experiments, the irradiated peroxidase was fractionated into monomers, dimers and higher aggregates, which were subsequently heated. The radiation-induced aggregates generally showed a higher heat-lability than the radiation-modified monomers. With increasing dose the heat-lability for all fractions was enhanced, which offers an explanation for the increase of the synergistic factor at higher doses on a combined treatment.

Table 2

*The effect of heating (5 min at 90 °C) on the monomers, dimers and higher aggregates isolated by preparative gel filtration of irradiated horse-radish peroxidase*

Residual activity before heating: 100%  
Protein: 0.1%

Fraction	Dose (Mrad)			
	0	0.5	1	2
	Residual activity in %			
Monomer	78	33	22	6.9
Dimer		27	10.0	5.0
Higher aggregates		16.5	12.4	3.9

In experiments with an isolated isoenzyme, that present in the highest amount in the isoenzyme mixture, we obtained similar results (Table 3). The activity measurements revealed that the sequence irradiation followed by heating was by far the more efficient treatment. Both sequences, however, showed synergistic effects. Whereas for the sequence heating followed by irradiation the synergistic factor  $F$  showed a constant value of about 2, for the sequence irradiation followed by heating with higher doses an increasing value was achieved, which varied from 6 at the lowest dose to 370 at the highest dose applied. The synergistic effect was more pronounced in the system of high purity, represented by an individual isoenzyme, as compared to the system of low purity in which the whole isoenzyme mixture with accompanying inactive proteins has been employed.

Thin-layer isoelectric focusing and thin-layer gel filtration of the isolated isoenzyme demonstrated again that heating before irradiation enhanced the relative amount of enzymatically active aggregates. For the sequence irradiation followed by heating the radiation-induced aggregates were preferentially inactivated by the subsequent heat treatment.



Table 3

*The effect of a combined irradiation and heat treatment on an individual isoenzyme (pI 7.1) of horse-radish peroxidase*

Protein: 0.1%

Treatment	min at 90 °C	Dose (Mrad)			
		0	0.1	0.2	0.4
		Residual activity in %			
1. irradiation	0	100	84	61	29
2. (irradiation + heating) calc.	5	75	63	46	22
3. heating + irradiation	5		30	20	12
4. irradiation + heating	5		10	1.0	0.06
		Synergistic factor (F)			
5. $\frac{(\text{irradiation} + \text{heating}) \text{ calc.}}{\text{heating} + \text{irradiation}}$			2.1	2.3	1.8
6. $\frac{(\text{irradiation} + \text{heating}) \text{ calc.}}{\text{irradiation} + \text{heating}}$			6.3	46	367

It would be very interesting to compare the sequence irradiation followed by heating with a treatment in which an equal amount of heat energy is applied in two steps, namely before and after irradiation. Such a treatment could be particularly efficient, because the pre-heating results in a higher percentage of radiation-induced enzymatically active aggregates, which in turn due to their higher heat-lability are easier inactivated by the subsequent post-heating. The total inactivation may be anticipated to be higher than on post-heating alone.

### 3. Conclusions

The experiments have shown that radiation-induced inactivation differs markedly from heat inactivation. On irradiation aggregation appears to proceed gradually through dimers, trimers, etc., which display noticeable enzyme activity, whereas the high-molecular aggregates formed on heating show only traces of enzyme activity. The changes in isoelectric pattern of monomers caused by irradiation are different from those caused by heat. The individual isoenzymes vary in their inactivation response. While the basic and acidic isoenzymes are most susceptible to irradiation, the acidic isoenzymes show the highest heat resistance. By the application of a combined treatment, the sequence irradiation followed by heating was synergistic and by far the more efficient treatment. The synergistic effect could be traced back to the increased heat-lability of the irradiated peroxidase, the radiation-induced aggregates being particularly heat-labile.



The aim of this study has been to provide a basis for a better understanding of the molecular processes which proceed during inactivation of enzymes in foodstuffs. We hope that further experiments will throw more light on the inactivation mechanisms and thus permit to achieve a better control of residual enzyme activity, which in turn should result in food products of better quality.

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## RADIATION PRESERVATION OF FRUIT JUICE SEMI-CONCENTRATES PREPARED BY CRYOCONCENTRATION\*

I. KISS

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The microbiological stability of fruit juices may be achieved by some physical or chemical processes, or by their combination. Cryoconcentration and treatment with ionizing radiations are physical processes which render the utilization of substances extraneous to food (preservatives) unnecessary and this is, from the point of view of food hygiene, extremely desirable. Studies were made to preserve strawberry, grape and apple juices by the combination of cryoconcentration and ionizing radiations. The fruit juice obtained by conventional methods was clarified with a pectolytic enzyme preparation and then concentrated to various solids content (30—50%) by means of cryoconcentration. The products were then irradiated with  $^{60}\text{Co}$  radioactive isotope in the dose range of 0.3 to 1.3 Mrad and stored at 20—22 °C.

During the storage period it was established that the microbiological stability and the organoleptic quality of the products are highly dependent on the water activity, on the temperature of clarification and on the radiation dose applied. The keeping quality of a product of 40% solids content was substantially extended with 0.6 Mrad and treatment with 0.8 to 1.3 Mrad ensured a practically stable product. The colour of the semi-concentrates faded as an effect of irradiation. During storage browning processes occurred and this was particularly striking in the case of strawberries. The organoleptic qualities of the 40% concentrate remain unchanged and it retains the original aroma.

Cryoconcentration is a promising new method of fruit juice processing. This method has the great advantage of not damaging the initial aroma, colour and nutrient content of the fruit. Thus the juice obtained by the dilution of a cryoconcentrate is practically equivalent to the fresh fruit juice (KÖLLÖ *et al.*, 1959; ERDÉLYI & TURJÁN, 1965; SMITH, 1965; DOOLEY & LINNEBERRY, 1966). However, complete microbiological stability is very difficult to achieve even by concentration. The keeping quality of the semi-concentrates gained by cryoconcentration is substantially better than that of the original juice (BEKE *et al.*, 1965; ALMÁSI, 1968). The production of concentrates of more than 50 per cent solids content is not economical, because the power requirement of ice separation increases with increasing concentration and at the same time a higher percentage of fruit solids adheres to the ice. To store the semi-concentrate for a longer period, it is necessary to apply refrigeration or chemical preservatives (BAJNOK & VAS, 1963).

\* Presented at the IUFOST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



Therefore we examined the effect of the ionizing radiation for extending the shelf-life of the semi-concentrates at room temperature.

## 1. Materials and methods

### 1.1. Preparation, cryoconcentration and storage of fruit juices

Preparation of samples was as follows:

The apples (Jonathan variety) were washed, then ground in a hammer mill and the juice was extracted with a filter press. The grapes (Riesling—Sylvaner variety) and the strawberries (Senga-Sengana variety) were pressed directly.

0.3 per cent of a Hungarian pectolytic enzyme preparation ("Polizim", Standard Pectolytic Activity,  $\text{SPA}_{75} = 2\,000\text{ l} \cdot \text{h}^{-1} \cdot \text{l}^{-1}$ ) was added to the juice. The apple juice was clarified at 45, 50 and 60 °C and the grape juice and strawberry juice at 45 and 55 °C for 1 h. The clear juice was separated from the sediment by centrifuging and concentrated by freezing out the water at -27 °C. The ice formed was separated by centrifuging.

The strawberry juice was concentrated to 30 per cent solids content, then made up to 50 per cent solids content by the addition of granulated sugar. The grape juice was concentrated to 41 per cent, the apple juice to 48 per cent solids content.

Both the irradiated and untreated samples were stored at 20–22 °C and compared in organoleptic tests to samples stored at -5 °C.

For sensory evaluation the samples were submitted to a panel of 10 judges for scoring. Kramer's method was applied for evaluation (KRAMER, 1960). The concentrates were diluted with tap-water to their respective original solids contents. The absorption spectra of the concentrates were established with the Perkin—Elmer spectrophotometer type 137.

### 1.2. Radiation treatment

The juice concentrates were irradiated in panoramic  $^{60}\text{Co}$  radiation sources of 1 500 Ci and 80 000 Ci activity, resp. The dose rates were 0.072 and 1.0 Mrad/h, respectively. The doses applied were: 0.30, 0.46, 0.55, 0.60, 0.87, 1.0 and 1.3 Mrad, respectively. Ferrosulfate and chlorobenzene were used for dosimetry.



## 2. Results

### 2.1. Strawberry juice

The microbiological stability of strawberry juice concentrate, stored at room temperature was substantially increased by treatments with 0.3 and 0.6 Mrad, and by increasing the clarification temperature from 45 to 55 °C about half of this radiation dose was sufficient to achieve the same keeping quality (Fig. 1).

The total viable cell count of the unspoiled samples was determined immediately upon irradiation and several times during storage. The increase in both radiation dose and clarification temperature reduced the cell count. During storage the logarithm of the cell count of the unspoiled samples was further reduced, as observed already in earlier experiments (Kiss *et al.*, 1969). This shows that, if osmotolerant strains are not present in the microbial flora surviving radiation treatment, the latter becomes gradually destroyed by the high osmotic pressure of the medium. Thus spoilage is caused by the growth of the radiation-surviving fraction of osmotolerant yeasts. If the juice is prepared of good quality fruit and the viable cell count can be reduced, or at least kept at the low initial value during clarification and concentration, probably the number of osmotolerant yeasts is also lower than in concentrates of high initial cell count.

Investigations proved that the loss of colour intensity of anthocyanin containing fruit juices during storage is inversely proportional to the radiation dose and storage time and at the same time browning occurs.

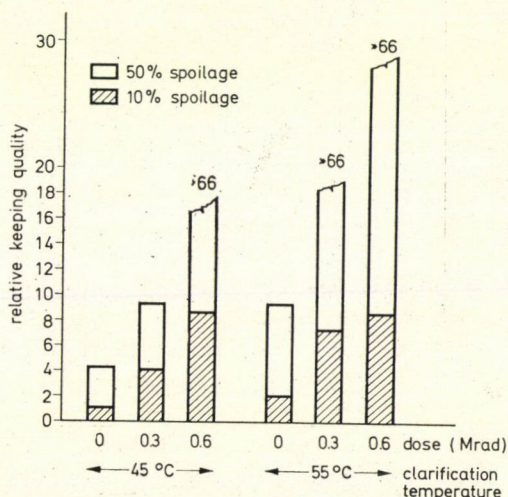


Fig. 1. Relative storage life of strawberry juice concentrate as a function of clarification temperature and radiation dose. (The relative storage life was determined by relating the spoilage time of samples to the unit: the time elapsing to 10 per cent spoilage level of the unirradiated samples of juices clarified at 45 °C.)



Thus the strawberry juice concentrates were, apart from the colour changes, of acceptable organoleptic quality even after a storage time of 5 weeks.

## 2.2. Apple juice

In earlier experiments it had been established that increased clarification temperature extended the storage life of strawberry juice, or reduced the irradiation dose requirement.

It was shown in the present study that clarification temperatures higher than the customary 45 °C, for instance 50 or 60 °C, increased the keeping quality of apple juice concentrate of 0.88–0.90 water activity. Apple juice concentrate prepared of juice clarified at 50 or 60 °C and treated with 1 Mrad did not spoil during four months storage at room temperature. The efficiency of the treatment is well demonstrated by Fig. 2 illustrating the relative keeping quality.

Concentrates of 0.90 water activity prepared of apple juice clarified at 45 °C, and treated with 1.3 Mrad were found unspilled after 4 years of storage at room temperature.

The microbiological stability, that is the total viable cell count during storage is very much dependent on water activity.

Irradiation (1 Mrad), however, reduced the viable cell count in the cryoconcentrate to a level, where, with the method applied, no viable cell could be demonstrated.

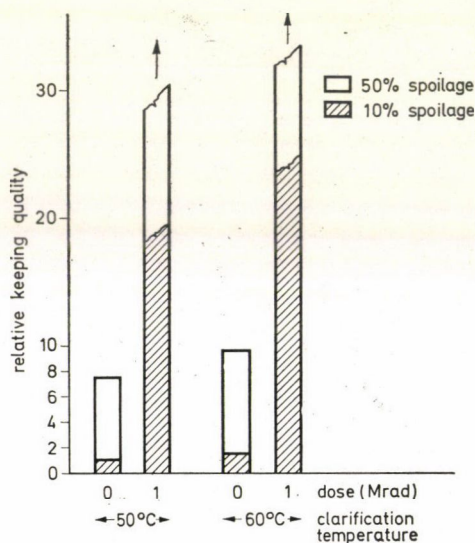


Fig. 2. Relative storage life of apple juice concentrate as a function of clarification temperature and radiation dose. (The relative storage life was determined by relating the spoilage time of samples to the unit: the time elapsing to 10 per cent spoilage level of the unirradiated samples of juices clarified at 50 °C.)

Due to the oxidation of various colour substances the colour of the fruit juice becomes darker. If irradiated, juices not containing anthocyanins get brighter, but during storage they behave like the juices not exposed to radiation and darken moderately.

On the basis of the organoleptic tests the conclusion was drawn that, of the characteristics observed, the general impression is formed mainly by the taste, therefore, in evaluating calculations, only the taste was taken into consideration.

It was established that except for one, all the average scores reached the acceptability limit. Comparing, at the 95 per cent probability level, the various treatments and storage temperatures, no significant differences were found (Fig. 3).

### 2.3. Grape juice

Ampoules are particularly suitable for the observation of spoilage symptoms. In these experiments we were able to establish the spoilage percentage with great accuracy. Grapes were harvested and pressed, the juice was clarified, cryoconcentrated, filled into ampoules and radiation treated, in

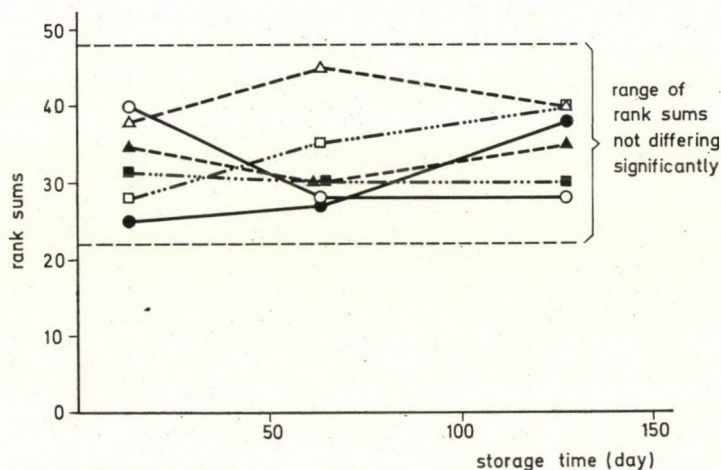


Fig. 3. Sensory evaluation of the taste of apple juice concentrate. The panel consisted of 10 panelists. The significance test was carried out at  $P = 5\%$  level. The dashed lines enclose the rank sums not differing

Storage temperature (°C)	Dose (Mrad)	Clarification temperature	
		50 °C	60 °C
-5	0	○	●
20	0	△	▲
20	1	□	■



rapid succession. Thus, initial total viable cell count could be kept at a low level. Clarification at 45 °C gave a viable cell count of  $10^4$ /ml, whereas treatment at 55 °C resulted in a cell count of  $10^2$ /ml.

The clarified samples subsequently treated with 0.87 Mrad showed no spoilage during the observation period of 18 months, thus the grape juice concentrate may be considered stable.

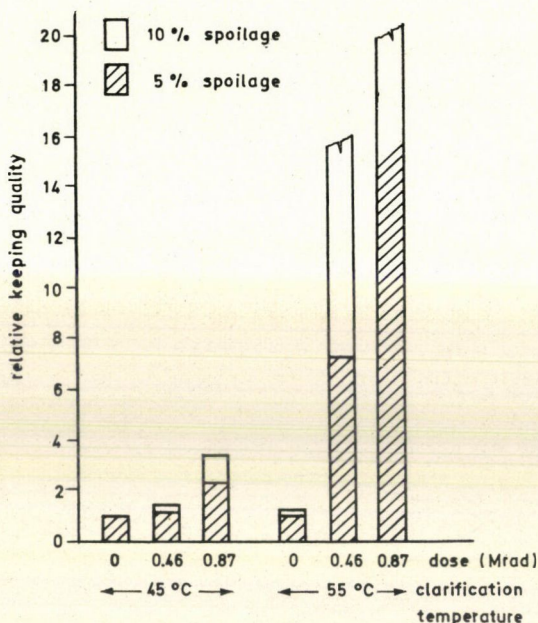


Fig. 4. Relative storage life of grape juice concentrate as a function of clarification temperature and radiation dose. (The relative storage life was determined by relating the spoilage time of samples to the unit: the time elapsing to 5 per cent spoilage level of the unirradiated samples of juices clarified at 45 °C.)

The efficiency of treatment is well demonstrated by Fig. 4 illustrating the relative keeping quality.

Taking 5 per cent spoilage as the basis, clarification at 55 °C and radiation treatment with 0.46 Mrad resulted in doubling the storage life, and applying 0.87 Mrad ensured at least 70-fold extension, as against the cryoconcentrate of juice clarified at 55 °C but not exposed to radiation treatment.

Various authors observed off-flavour in juices of low solids content and treated at a high dose level (Kovács, 1969). No such observation was made with the concentrates. To establish whether this phenomenon occurs in the diluted concentrates, the concentrate was diluted to 15, 30 and 41 per cent solids content, respectively, prior to radiation treatment.



In the sensory tests, score 3 was considered the lowest level of acceptability and in accordance with this, the grape juice of 15 per cent solids content was found not acceptable 24 h after treatment with 0.87 Mrad. However, after a storage time of 11 days it reached the 3-score level. This means that the off-odour was eliminated by that time. On the eleventh day the samples of 15 and 30 per cent solids content were fermented and could not therefore be judged, and the concentrate of lowest water activity was of inferior quality. The same was proven by the rank sums and the two samples differed significantly from the rest at 95 per cent probability level and were worse than the rest (Fig. 5).

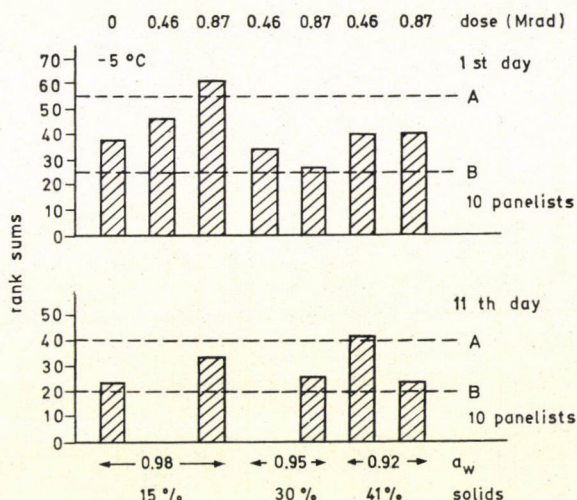


Fig. 5. Sensory values as obtained in grape juice concentrate as a function of rank sums, solids content and radiation dose. (The untreated samples were stored at  $-5^{\circ}\text{C}$ , the treated ones at  $20^{\circ}\text{C}$ ,  $a_w$  = water activity.)

The colour of juices containing anthocyanin pigment (strawberry) was not affected by clarification at  $55^{\circ}\text{C}$ . Probably this temperature does not inhibit the activity of the dehydrating enzyme. Polyphenols turn brown in the presence of oxygen and this is an enzymic dehydration process (WEINGES *et al.*, 1968, 1969). Our experience was that the juice clarified at  $45^{\circ}\text{C}$  and its concentrate was darker than those treated at  $55^{\circ}\text{C}$ . The difference between the two samples in the process of browning proved constant. Treatment with 0.87 Mrad had a stronger browning effect than with 0.46 Mrad.

Results permit the conclusion that the enzymes catalyzing the oxidation of colour substances are inactivated, at least partially, by a treatment at  $55^{\circ}\text{C}$ .



### 3. Conclusions

Cryoconcentrated fruit juices may be stored for longer periods only at low temperature or on addition of chemical preservatives. When combined with ionizing radiation the method permits the storage of fruit juices with relatively low solids content (40 per cent) at room temperature (20–22 °C).

The microbiological stability of the concentrates is highly increased by treatment with 0.6 Mrad, and 0.8–1.3 Mrad practically ensures stability. It was established that the application of enzymic pectolysis at 55 °C reduces the viable cell count of the juice and this allows a reduction of the dose requirement.

The water activity of the juice significantly influences the sensory qualities. Changes in palatability as observed by other authors were found only in juices of high water activity (0.96–0.98). These off-odours and off-flavours disappeared in a relatively short time. The colour of the juices suffers a change during storage. The anthocyanins are radiation sensitive and darkening, browning occurs. In apple juice an initial brightening of colour was observed upon radiation treatment, however, later it redarkened.

On the basis of the results it was established that cryoconcentrated fruit juices of 40 per cent or higher solids content reach microbiological stability if treated with 0.8–1.3 Mrad and thus may be stored at 20–22 °C. The dose requirement may be reduced by increasing the clarification temperature of the fresh juice from 45 °C to 55 °C. The sensory quality of the radiation treated juice is satisfactory.

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## ANTIBIOTIC, INHIBITORY AND TOXIC METABOLITES ELABORATED BY MICROORGANISMS IN FOODS\*

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Possible use of antibiotics in combination with, or instead of, heat to control spoilage led to early applications that involved subtilin, nisin, tylosin, oxytetracycline, and chlortetracycline in canned goods, cheese, meats, poultry, fish and other foods. The inhibitory activity of these antibiotics was sufficient to destroy many food spoilage organisms but the development of resistant (*R*-factor) strains limited their usefulness. The antagonism of selected *Enterobacteriaceae*, *Streptococci* and other lactic acid bacteria has established the preservative value of bacteriocins. In contrast to these beneficial applications, the development of toxic metabolites such as botulin, enterotoxin, aflatoxins, ochratoxins, tricothecenes, and psoralens has limited the use of biological entities as food preserving agents. Various antibiotic, inhibitory and toxic metabolites elaborated by microorganisms growing in or on foods are cited and an appraisal of their benefits, and limitations are presented.

### 1. Antibiotics

FLEMING's (1929) discovery of penicillin and its rediscovery as a therapeutic agent by CHAIN *et al.* (1940) and ABRAHAM *et al.* (1941) during World War II were instrumental in establishing the value of antibiotics in combating bacterial infections in man. As a result of the success that attended the introduction of antibiotics in the field of medicine, interest quickly developed concerning the possibility of using these agents for preserving foods (Table 1).

Table 1  
*Antibiotics used in foods*

subtilin  
nisin  
tylosin  
chlortetracycline  
oxytetracycline  
chloramphenicol  
nystatin  
streptomycin  
bacitracin  
rimocidin  
ascosin  
pimaricin

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CURRAN and EVANS (1945) observed a marked reduction in the number of viable spores of three species of *Bacillus* in milk with as little as 5 Oxford units per ml of penicillin. In later papers they (CURRAN & EVANS, 1946a, b) indicated that neither penicillin nor streptomycin proved to be practical agents for preserving foods since their activity against bacterial spores was quite limited. Similarly, TARR and DEAS (1948) reported that penicillin and streptomycin were of relatively little value as preservatives for fresh fish.

### 1.1. Use of antibiotics and mild heat

*Subtilin.* As a consequence of reports issued from the Western Utilization Research and Development Division of the U.S.D.A. in 1949 and 1950, much interest was aroused in the possible use of antibiotics as food preservatives. ANDERSEN and MICHENER (1950a, b) reported that subtilin in low concentrations in combination with mild heat had a marked preservative effect when used in a variety of canned foods. A cursory study by HOUNIE (1950) suggested that a mixture of subtilin and streptomycin might prove useful with meat. He noted that ground meat inoculated with *Clostridium* sp. kept for 5–10 days in the presence of a mixture of these two antibiotics. However, ADAMS *et al.* (1951, 1952) and CAMERON and BOHRER (1951) found subtilin useless at commercially practical levels; the first group of workers encountered almost complete spoilage of comminuted beef containing *Clostridium* sp. PA 3679 while CAMERON and BOHRER reported that subtilin was completely ineffective against some strains of *Clostridium botulinum*. Similarly, BURROUGHS and WHEATON (1951) found that bacitracin, gramicidin and streptomycin as well as subtilin, did not prevent the development of *Clostridium botulinum* in canned peas or corn. EVANS and CURRAN (1952), after finding that spoilage consistently occurred in normal or concentrated milk inoculated with spoilage organisms and subtilin, concluded that the subtilin-heat process was not a safe or effective method for preserving these products.

*Nisin.* Most of the better-known non-toxic antibiotics have been tested for use in foods. Nisin, a metabolic product of *Streptococcus lactis* and produced naturally during milk souring, has been widely used for the preservation of cheese and processed cheese. As had been observed with subtilin, nisin allowed spores to germinate but prevented lysis or outgrowth of the spore wall. Spores in which outgrowth was normally by rupture of the spore wall, were more sensitive than those in which the cell wall was lysed (GOULD, 1964). Less than 10 Reading Units of nisin per ml (RU/ml) inhibited sensitive spores but resistant spores developed in over 100 RU/ml. The activity of nisin was so reduced during pressure cooking that it did not control mesophilic spoilage.

*Tylosin.* The macrolide, tylosin, was considered more heat resistant than subtilin or nisin. DENNY *et al.* (1961a, b), GREENBERG and SILLIKER (1962a, b, c),



SHENEMAN (1964), WHEATON and HAYS (1964) and MALIN and GREENBERG (1964) presented studies on the role that tylosin plays in controlling spoilage in various foods including mushrooms, ice cream mix, processed cheese, ham, sausage, beef-steak and gravy, chop suey, spaghetti and meat balls, corned beef hash, chili, cream style corn, chicken chow mein, cream of chicken soup, tuna and noodle dinner, and smoked fish. Tylosin has the advantage that its action depends upon inhibiting cell growth rather than spore germination. While tylosin did not prevent germination even at 100 times the minimum growth inhibitory concentration, vegetative cells outgrowing from spores swelled and elongated but did not divide. With high concentrations of tylosin ( $>5 \mu\text{g/ml}$ ) spores did not shed the spore wall (GREENBERG & SILLIKER, 1962b; GOULD, 1964).

### 1.2. Extending storage life of flesh foods and other food products

In 1950, TARR *et al.* published a note and later (1952) a paper in which they reported that of 15 antibiotics investigated, only three, chlortetracycline (CTC), oxytetracycline (OTC) and chloramphenicol (Chloromycetin, CA), were effective in preserving fish and beef. CTC was considered the most active of the three. With this antibiotic, however, yeast developed on the treated samples. In experiments wherein rimocidin was included, development of yeast was markedly retarded over that which occurred when CTC was used alone. Results obtained by GOLDBERG *et al.* (1953) on fresh ground beef with six antibiotics — penicillin, streptomycin, bacitracin, chlortetracycline, chloromycetin and oxytetracycline — agreed quite closely with those reported by TARR *et al.* (1952); CTC, OTC and CA were the most effective preservatives for meat. Later, WEISER *et al.* (1953) observed that there was less deep spoilage in beef carcasses infused with CTC than in the controls but noted that those tissues having the greatest blood supply also contained the highest levels of antibiotics. LEPOVETSKY *et al.* (1953) suggested that organisms present in the lymph nodes are released after death and ultimately contaminate the deep tissue.

WEISER *et al.* (1953) showed that, during storage of ground beef, Gram-negative rods predominated but that CTC and OTC retarded their development. Later JAY *et al.* (1956) demonstrated that certain strains of *Proteus* and *Pseudomonas* were inhibited by antibiotics in fresh beef. On the other hand, AYRES *et al.* (1956) showed that 65% of the flora on raw poultry after two days of storage under normal conditions consisted of *Pseudomonas* and *Achromobacter* but after treatment with 10 ppm CTC, during the same period of storage, 95% of the organisms belonged to these groups. There was also a significant increase in the numbers of yeasts in the population while under treatment with antibiotics. Other workers have confirmed these findings under different conditions (EKLUND *et al.*, 1956; NJOKU-OBI *et al.*, 1957; BARNES & SHRIMPTON, 1959).



*Tetracyclines.* In the mid 1950's the tetracyclines, chlortetracycline and oxytetracycline, were found superior to other antibiotics for doubling the storage life of meats, fish and poultry and, in 1955 (CTC) and 1956 (OTC), the U.S. Food and Drug Administration approved their use in ice or cooling water at a residual level not exceeding 7 ppm for preserving dressed poultry. In 1959 a tolerance of 5 ppm was allowed on fresh fish and shellfish. Difficulties were experienced when results obtained in the laboratory were translated into commercial practice.

Studies at the Torrey Research Station indicated that there was an initial reduction of the pseudomonad portion of the flora and a continued resistance to its re-establishment. This was gradually overcome by the development of resistant strains which finally became the major portion of the total flora (LISTON *et al.*, 1957). The work of BRANDES (1958) also indicated the selection of a specialized flora consisting mainly of pseudomonads during spoilage of fish stored in ice containing antibiotics. DE SILVA (1964) found that pseudomonad domination occurred earlier during oxytetracycline-treatment than when birds were normally handled. This was not so with CTC-treated fish; however, achromobacters were an important constituent of the treated fish. During both types of antibiotic treatments there was a marked increase in the numbers of fluorescent types of pseudomonads.

The tendency for those in the food industry to use antibiotics as a substitute for good manufacturing practices and the marked chelating ability of the tetracyclines in the presence of iron (esp. when rusty equipment was used) negated the value of these antibiotics. Further, the possibility that resistant or *R*-factor pathogenic microorganisms might persist and that other organisms might develop which were previously not associated with spoilage, prompted federal regulatory officials to rescind permission to use antibiotic dips.

*Macrolide antibiotics.* The macrolide antibiotics, rimocidin and pimaricin possess antimycotic properties toward many of the yeasts and molds found in and on food products (KLIS *et al.*, 1959). In early studies, TARR *et al.* (1952) found rimocidin useful in preventing yeast growth on fish treated with chlortetracycline (CTC), and AYRES *et al.* (1956) reported that both rimocidin and pimaricin inhibited the development of yeasts indigenous to dressed chicken. When used alone in water rinses at levels of 10 µg/ml, pimaricin or rimocidin held the total flora in check for 5–7 days and prevented yeast proliferation for 12 days or more; two macrocyclic lactones, ascocin and nystatin, did not. Interest in rimocidin waned when it was reported (DAVISSON *et al.*, 1951) that the compound was hemolytic to human red blood cells and toxic to mice. Nystatin (ANON, 1957) and pimaricin (ANON, 1963) were reported to be non-toxic and without allergic response when taken orally by human subjects. LEVINSKAS *et al.* (1964) found only minor differences in food intake and weight gains when levels of as high as 1 000 µg/g of pimaricin were fed to rats for 2 years



and there were no differences in reproductive performance of control rats and those fed diets containing 1 000  $\mu\text{g/g}$  pimaricin. Also, according to CLARK *et al.* (1964), two-year toxicity studies in rats and dogs were completed with no untoward effects.

YACOWITZ *et al.* (1957) indicated that when nystatin was used in conjunction with CTC, it effectively prevented growth of yeasts and molds on stored chicken wings and drumsticks held for at least 7 days. AYRES and DENISEN (1958) observed that growth of microorganisms on strawberries, raspberries and cranberries dipped in water containing 10  $\mu\text{g}$  antibiotic/ml, was delayed when pimaricin or rimocidin was used but enhanced with candidin or ascocin; nystatin had little preservative effect.

With pimaricin the slope of the growth cycle for yeasts and molds flattened as the amount of antifungal agent was increased in the range of 10, 20, 50 and 100  $\mu\text{g/ml}$  (AYRES *et al.*, 1964). The action on yeasts was fungistatic and, upon dilution, there was outgrowth even at 100  $\mu\text{g/ml}$ . Berries sprayed with 50 or 100  $\mu\text{g/ml}$  nystatin, pimaricin or rimocidin and allowed to dry prior to harvesting had improved storage life, but percentages of sound fruit were less than for lab-treated berries. Tomatoes sprayed in the field or dipped post-harvest showed no improvement in storage life over untreated controls. The tomato cuticle was not adequately wetted by the spray and while the addition of spreader-stickers in the spray solution resulted in adherence, the storage life of the fruit was not extended. Nystatin or pimaricin applied at weekly intervals during the growing season also was without effect; the antibiotics were destroyed in the presence of sunlight, and examination for residual amounts were negative after only a short exposure time.

WHITEHILL (1956; see CLARK *et al.*, 1964) showed pimaricin to be effective in retarding the growth of yeasts and molds in cottage cheese at 2.5 °C and suspensions of up to 100  $\mu\text{g}$  pimaricin/ml were reported by MOL (1959) to be useful for dip-treating hard cheese. SHAHANI *et al.* (1959) described the use of pimaricin and nystatin in controlling yeasts and molds in cheese and cottage cheese. Later, NILSON *et al.* (1960) reported that cheese washed with the antibiotic suspensions (20 to 100  $\mu\text{g/ml}$ ) in the second wash water contained 5–10  $\mu\text{g}$  of antibiotic per gram of cheese but that the antibiotics lost their activity completely in 3–16 days. Pimaricin was more effective than nystatin in retarding fungal spoilage. VAN EEK (1964) indicated that it was common practice by the cheese industry of the Netherlands to use 100  $\mu\text{g/ml}$  pimaricin solutions to avoid mold development on hard cheese surfaces.

SHIRK and CLARK (1963) evaluated the effect of pimaricin in retarding yeast spoilage of artificially inoculated and uninoculated fresh orange juice. Juice inoculated with natural contaminants spoiled after 1 week storage, while none of the lots containing 1.25, 2.5, 5, 10 or 20  $\mu\text{g/ml}$  of added pimaricin spoiled in 8 weeks.



While several of the antibiotics — and particularly nisin, tylosin, CTC, OTC, nystatin and pimarin — have proved to effectively inhibit bacterial proliferation, the use of these agents as food additives is viewed with suspicion by public health officials. Consumption of residual antibiotic present in food is considered undesirable since such practice might cause sensitization of the consumer to the antibiotic, alter the normal intestinal flora, allow resistant strains to persist and, in the event that competitive microbial forms are destroyed, an artificial selection of pathogens might be promoted. At present there is little activity insofar as the use of antibiotics for preserving foods is concerned.

## 2. Bacteriocins

Bacteriocins comprise a natural class of antibiotics produced by bacteria and distinguished from other antibiotics by their very narrow range of antimicrobial activity; they act only on strains of the same or closely related species. According to REEVES (1965), they are protein in nature. Bacteriocins are thought to destroy after first adsorbing onto a specific receptor, such as that of an antibody or an antigen, with mutation to bacteriocin resistance involving loss of the receptor. Some act only on a few strains while others have a wider range of action.

Bacteriocinogeny can be transferred from bacteriocin strains to others of the same type through cell contact. Bacteriocinogenic strains do not always produce bacteriocins; various agents either induce the organisms or act as carcinogens or mutagens (FREDERICQ, 1963a).

GRATIA (1925) initiated the study of bacteriocins when he observed inhibition of *Escherichia coli*  $\Phi$  by *E. coli* *V*. His careful studies and later those of FREDERICQ (1948, 1957, 1963b) led to much of our present knowledge on bacteriocins and the use of colicins as a model for classifying other bacteriocins. There are various families of bacteriocins (Table 2) including: Colicins, Alveicins, Carotoviricins, Arizonacins, Cloacins, Marcescins, Pneumocins, Aerocins, Pyocins, Fluocins, Pesticins, Megacins, Monocins, Cerecins, Enterococcins, Staphylococcins, and a miscellany of others. According to HAMON and PÉRON (1963), every group of the *Enterobacteriaceae* except *Providencia-Providencia* can produce bacteriocins.

*Colicins*. Colicins are grouped according to their bactericidal specificity (IVANOVICS, 1962). From 20 to 25% of producing strains are found in *E. coli*. Other producers include *E. freundii*, *Paracolobactrum*, *Shigella* and less frequently *Salmonella* and *Aerobacter*.

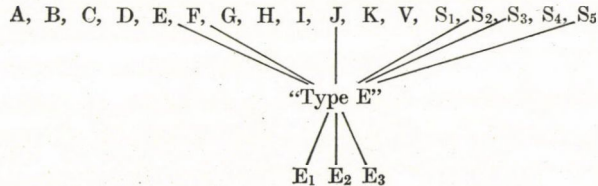
FREDERICQ (1946) observed that colicin resistant cells grew out whenever sensitive cells were treated with colicin; yet the cells were sensitive to many other colicins. By using these mutants, he was able to segregate them into 17



Table 2

*Bacteriocins*

colicins — *Escherichia coli*; *E. freundii*; *Paracolobactrum*; *Shigella*; *Salmonella*; *Aerobacter*; *Serratia* (?)



alveicins — *Hafnia*; majority have affinity with colicin E

carotovoricins — *Erwinia*

arizonacins — *P. arizonae*; some sensitive to colicin E

cloacins — *Enterobacter cloacae*

marcescins — *Serratia*; mostly *S. marcescens*

pneumocins — *Klebsiella*; mostly *K. pneumoniae*

aerocins — *Aerobacter aerogenes*

pyocins — *Pseudomonas pyocyanea*

fluocins — *Pseudomonas fluorescens*; other *Pseudomonas*

pesticins — *Pasteurella pestis*

megacins — *Bacillus megaterium*

monocins — *Listeria monocytogenes*; types A and B

cerecins — *Bacillus cereus*

enterococcins — all *Streptococcus zymogenes* — type 1

*S. liquefaciens* — type 2

some strains: *S. faecalis* and *S. faecium* — type 3

some strains of *S. faecium* — type 4

one strain of *S. zymogenes* — type 5

staphylococcins — *Staphylococcus*; 5 types

types, *i.e.* A, B, C, D, E, F, G, H, I, J, K, V, S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, S<sub>4</sub> and S<sub>5</sub>. Since then, colicins have been discovered which do not fit Fredericq's original classification (FREDERICQ, 1951; PAPAVALASSIOU, 1961). FREDERICQ (1950) amalgamated E, F, J, S<sub>2</sub>, S<sub>3</sub> and S<sub>5</sub> into a new type E which he subsequently (1956a) subdivided into E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub>. HAMAN and PÉRON (1963) refer to 23 different types.

Within each species only certain colicin types were produced (FREDERICQ, 1948). For example, *E. freundii* produced type A only; *E. coli* produced types B, C, D, E, F, G, H, I or V; *Paracolobactrum* produced J or K; *Shigella* produced S<sub>1-5</sub> and *Salmonella* produced I, K or B (FREDERICQ, 1963b). In general, a mutant resistant to one colicin was resistant to all of that group but not necessarily to colicins of other types.

Colicins are thought to adsorb from solution by sensitive bacteria onto specific receptors on the cell surface and so by Fredericq's classification they fit into groups according to the receptor onto which they adsorb (REEVES, 1965). FREDERICQ (1954, 1956a, b, 1963c) believes that colicin production is determined by plasmids, genetic determinants not associated with the chromosome, which he terms C-factors. A C-factor, in addition to determining ability



to produce colicin, also confers immunity to that colicin and to others of the same type but such immunity may not be complete. Since the immunity does not involve receptor loss, it resembles the immunity conferred by a prophage against superinfection by the same or a closely related phage (REEVES, 1965).

Several strains of *E. coli* antagonistic toward contaminating *Salmonellae* were tested in fermenting egg white by FLIPPEN and MICKELSON (1960). Two of the strains exerted strong inhibitory action on *S. senftenberg*, *S. oranienburg* and *S. typhimurium*. The most efficient *Salmonella* antagonist, *E. coli* 6-204-55, successfully desugared the egg white and was not enteropathogenic (MICKELSON and FLIPPEN, 1960). The destruction of *Salmonella* cells was not complete even after a 48-hour fermentation period but when 0.5%  $H_2O_2$  was added, the egg-white was freed of the pathogen. This application of colicins provides a novel approach in producing *Salmonella*-free food products.

*Enterococcin*. BROCK *et al.* (1963) found that over 50% of 99 strains of various species of enterococci produced bacteriocins and that these bacteriocins could be segregated into five distinct types on the basis of their activity spectra and sensitivity to chloroform, heat and proteolytic enzymes. According to these workers, the first type (Type 1) was produced by all strains of *Streptococcus zymogenes*. Type 1, in addition to being active against a wide variety of Gram-positive bacteria, is also a hemolysin. Type 2 is produced by some strains of *S. liquefaciens* and acts on all strains of *S. faecium* and some strains of *S. faecalis*. Type 3 is produced by certain strains of *S. faecalis* and *S. faecium* and inhibits a number of group D streptococci; is inactive against other lactic acid bacteria except for *Leuconostoc citrovorum*. Type 4 is produced by certain *S. faecium* strains and resembles Type 3 in activity but the two types do not always exhibit reciprocal cross resistance. Type 5 is produced by only one proteolytic strain of *S. zymogenes* and has a very narrow spectrum. None of the five types is sensitive to its own bacteriocin.

KAFEL and AYRES (1969) noted that enterococci acted antagonistically on some undesired bacteria and, particularly on selected species of *Clostridium*, *Bacillus* and *Lactobacillus* in a canned ham environment. (Figs. 1 and 2.) The organisms were able to grow at refrigeration temperature and had greatest antagonism for *C. perfringens*, *C. botulinum* A and E and *C. sporogenes*. Presumptive evidence indicated that the antagonistic agent was non-filterable and was inactivated by autoclaving. Inhibition was presumed to be due to the antibiotic nisin but bacteriocin types 3 and 4 have similar structural properties. Since types 1, 2 and 5 are insensitive to proteolytic action, work needs to be undertaken to determine if the material can be isolated by dialysis and, if so, the nature of its activity.

*Other bacteriocins*. The extremely selective action of most of the remaining bacteriocins limit their application to specific therapeutic measures; to date none of these pertain to foods.



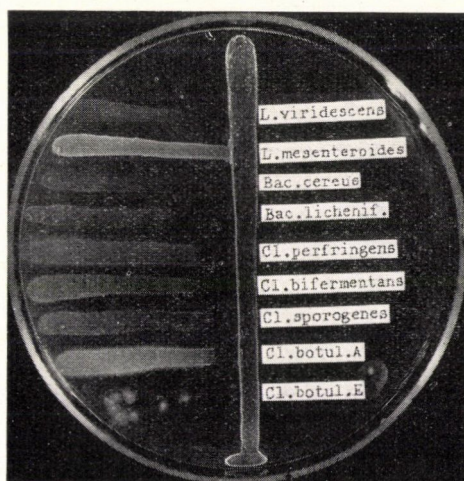


Fig. 1. Growth of various *Clostridia*, *Bacilli*, and *Lactobacilli* on APT agar in competition with enterococci (placed along the diameter of the plate)

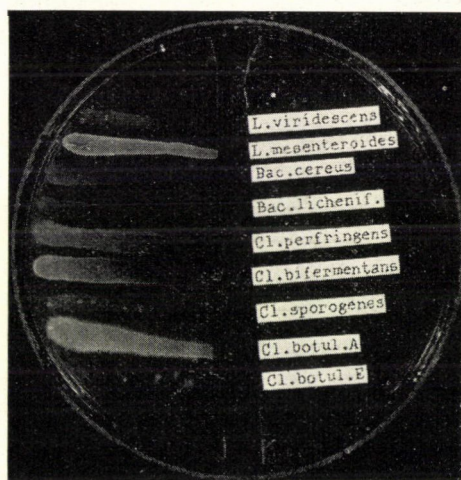


Fig. 2. Growth of various *Clostridia*, *Bacilli*, and *Lactobacilli* on APT agar after removal of growth of an enterococcal inoculum along the diameter of the plate

### 3. Botulin

The exotoxin of *Clostridium botulinum* is becoming known as botulin. There are six types of botulin toxins *i.e.* types A, B, C, D, E, and F, produced by different strains of *C. botulinum*. These toxins are antigenic, thermolabile exotoxins and are simple proteins held within a complex that is easily broken



by physical forces. Botulinal toxins A, B, and E are aggregates that contain toxic and non-toxic entities and their potency increases by separating the toxic moiety from the non-toxic component.

Crystalline type A botulinal toxin, with a molecular weight of 900 000 is separable into a neurotoxin having a molecular weight of 150 000 and a non-toxic hemagglutinin. Upon treating with proteolytic enzymes, crystalline toxin loses toxicity but not hemagglutinating activity (MEYER & LAMANNA, 1959). Type B toxin exists as a protein with a molecular weight of 165 000 and appears to be an aggregate of separable toxic and hemagglutinating proteins. Type E toxin, with a molecular weight of 350 000 (KITAMURA *et al.*, 1968), also exists as an aggregate of different simple protein components and is separable into toxic and non-toxic non-hemagglutinating components of approximately similar dimensions. The toxic moiety of type E toxin increases in biological activity after a short exposure to trypsins. The structures of types C, D and F toxins is less well known.

Botulism, while a highly fatal toxicosis, occurs rarely. In 1963 there was an unusually high incidence (47 cases) in the U.S., but since 1965 only 5–16 cases have been reported annually. Meats, fish, seafood, milk products and other low — and medium — acid canned foods are the products that have often been involved in botulism outbreaks. The most recent fatality from a commercial product resulted (1971) from the consumption of a can of underprocessed vichyssoise.

Temperature and pH are important factors in determining if toxin production will take place in foods. Ordinarily, pH 4.8 is a limiting pH for growth and spores are not known to germinate below pH 4.6. The various *C. botulinum* types differ in their temperature requirement. The lowest temperature for spore germination of types A and B is about 15 °C and their maximum temperature for growth is about 48 °C. In contrast to these, *C. botulinum* type E cells are able to produce gas and toxin at as low as 3.3 °C and their maximal growth temperature is about 45 °C. The heat resistance of spores of types A and B is much higher than for C, D and E; for example, type A spores can survive for 300 minutes at 100 °C or for 2.8 minutes at 121 °C while spores of type E are inactivated within 15 minutes at 80 °C.

#### 4. Enterotoxin

Under certain conditions *Staphylococcus aureus* produces a toxic protein called enterotoxin which, upon ingestion, elicits an emetic response in cats, dogs, monkeys and man. There are at least four immunologically distinct enterotoxins, A, B, C and D. All are simple proteins having similar physical properties and amino acid compositions and molecular weights in the range



30 000—35 000 (FOSTER & BERGDOLL, 1968). In addition, enterotoxin E has been tentatively identified. Many of the responses in animals to enterotoxin are similar to those induced by endotoxins (BERGDOLL, 1970).

While the enterotoxins are considered resistant to heat, they vary in their response. A is the most sensitive, showing a 50% decrease in reaction within 20 minutes at 60 °C and complete inactivation after 1 minute at 100 °C while B retains half its activity after heating at 60 °C for as long as 16 hours and for 5 minutes at 100 °C (BERGDOLL, 1967).

Staphylococcal food poisoning is not a reportable disease in the U.S. but in the past several years has accounted for about one-fourth of foodborne disease outbreaks. Enterotoxin A is the most frequently involved in such outbreaks. In almost half of 75 different incidents, 49% of the staphylococci isolated produced A alone, 29% produced a mixture of A and some other type, 10% produced D, and B and C comprised the remainder (CASMAN, 1967).

A wide assortment of animals have been tried experimentally but most are not susceptible. The monkey is considered to be the most reliable (SURGALLA *et al.*, 1953). Man is estimated to be much more sensitive to enterotoxin than any of these animals. There is evidence that less than 1 µg of enterotoxin A or 0.015 µg/kg may cause illness in humans while 2 µg/kg is required for emesis in the monkey (BERGDOLL, 1969).

Staphylococcal food poisoning is generally associated with foods that have been heated, then contaminated and kept unrefrigerated, for several hours. These organisms do not usually gain ascendancy in raw foods, but since they can persist at lower water activity ( $a_w$  0.86) than most microorganisms, they survive in salted as well as in cooked foods. Fillings in bakery goods, salads, salad dressing, puddings, cream sauces, cooked poultry-, egg-, meat- and fish-products (*e.g.* roast fowl, egg salad, ham, dry sausage, and seafood dishes) and milk products (cheese) have been responsible for many outbreaks. Food handlers are a prime source of contamination. Other sources include animals, personnel and contaminated equipment used in food production and processing. For example, mastitic cows contribute a continuing load of staphylococci that contaminate and re-contaminate milk products, individuals involved in milk production and processing and holding vats, separators, pasteurizers, clothing and other fomites.

## 5. Mycotoxins

A severe and widespread epidemic of man and animals occurring in the Orenburg district of the USSR from 1942 to 1947 was related to the consumption of moldy millet. In 1944 many fatalities occurred and alimentary toxic aleukia (ATA) or septic angina affected more than 10 per cent of the population



consuming cut—but unharvested—grain that had overwintered in the fields (JOFFE, 1965). The production of toxin was associated with fungal growth on the grain at low temperature. Numerous isolates from the genera *Fusarium*, *Cladosporium*, *Alternaria*, *Penicillium*, and *Mucor* were associated with the grain.

*Tricothecenes or T-2 toxins.* The genera *Fusarium*, *Trichothecium*, *Myrothecium*, and *Cephalosporium* produce highly toxic scirpene metabolites, called 12,13-epoxy- $\Delta^9$ -tricothecenes or T-2 toxins, at low temperatures (BAMBURG & STRONG, 1971).

According to SNYDER and HANSEN (1945) *F. tricinctum*, *F. poae*, *F. chlamydosporium* and *F. sporotrichioides* are synonymous and *F. nivale* is similar morphologically. Types of responses to the tricothecenes suggest that these compounds may cause ATA (BAMBURG & STRONG, 1971). ATA may progress in four stages from a burning sensation due to local irritation of mucous membranes of the buccal cavity and gastro-intestinal tract followed by a leukopenic stage and then to a sudden and severe hemorrhagic diathesis with anemia, increased leukopenia and necrosis, sepsis, exhaustion of the bone marrow and if death did not ensue, final convalescence (JOFFE, 1971).

*Psoralens.* Furanocoumarin compounds, more commonly known as psoralens, and which occur in the leaves, stems, roots and fruit of certain plants, have been reported to be responsible for a dermatitis in man. A blistering skin disorder is common among celery workers coming into contact with plants infected by the fungus *Sclerotinia sclerotiorum* (BIRMINGHAM *et al.*, 1961; PERONE *et al.*, 1964). A necessary condition for such lesions is that, following mold contact, the skin must also be exposed to sunlight or to ultraviolet radiation in the range of 320–380 nm. Without such exposure within a few minutes of the time of contamination, lesions are not produced.

SCHEEL *et al.* (1963) isolated and characterized two phototoxic furanocoumarins, 8-methoxypsoralen (xanthotoxin) and 4,5,8-trimethylpsoralen, from diseased celery (pink rot) but did not detect these compounds in undiseased plants. Some years earlier 5-methoxypsoralen, a phototoxic furocoumarin called bergapten had been isolated from celery (MUSAJO *et al.*, 1954). Since the mold *Sclerotinia sclerotiorum* can attack almost any kind of horticultural crop, it is one of the most widely distributed pathogens in the vegetable world. Recent work (WU *et al.*, 1972) has shown that, under experimental conditions, xanthotoxin and bergapten were produced by the fungus only in the rotted areas and that for production of the two psoralens, the concurrent metabolism of celery and of *S. sclerotiorum* is required. These compounds were not produced on celery or on artificial media at a room temperature lower than 15 °C nor were they formed on the non-metabolizing vegetable. Similarly, although the mold grew well on carrots, sweet potatoes, artichokes, oranges, cucumbers, and turnips, no psoralen could be detected.



In England, in 1960, a disorder, called "Turkey X Disease", involving the loss of turkey poults fed moldy peanut meal, was reported (BLOUNT, 1961). The toxicity was not limited to turkeys; there were also outbreaks of disease in ducklings, pigs and calves (ALLCROFT, 1965). Severe losses of ducklings from a similar disease occurred in Kenya and Uganda (ASPLIN & CARNAGHAN, 1961).

Also in 1960, hepatomas of hatchery-reared rainbow trout appeared in epidemic proportion in private, state, and federal hatcheries in the United States and Europe following the introduction of dry, pelleted commercial feeds (HALVER & MITCHELL, 1967).

*Aflatoxin.* It was soon learned that certain strains of the common mold *Aspergillus flavus* that grew in damp peanut meal or cottonseed products were able to synthesize highly substituted coumarins containing bifuran rings and lactone configurations (ASAO *et al.*, 1963). Their blue or green fluorescence, when exposed to ultraviolet light, caused these components to be designated B<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub>, G<sub>2</sub>.

Trout are unusually sensitive to aflatoxin. About 1/3 of a rainbow trout population developed hepatoma within a year when fed 0.5 ppb aflatoxin. Also, after 20 months on test, trout receiving 2 ppb B<sub>1</sub> had 60–70% tumour incidence and those receiving 20 ppb had 90–100%.

The first report of the detection of aflatoxins in human tissues was made by SHANK *et al.* (1971). They reported up to 93 µg B<sub>1</sub>/kg in liver tissue, 123 µg/kg in stool, 127 µg/kg in stomach and intestinal contents and 8 µg/kg in bile. Trace amounts were also detected in brain, kidney and urine. Further, they indicated that several hundred children in Thailand between the ages of 1 and 13 years died of a disease of unknown etiology. The disease was manifested in hospital cases by vomiting, convulsions and coma, and resulted in 80% deaths. Chemical analysis of autopsy specimens from 23 Thai children who died with acute encephalopathy and fatty degeneration of the viscera (EFDV) revealed aflatoxin B<sub>1</sub> in one or more specimens from 22 of the 23 EFDV cases. The extent of aflatoxin contamination in food samples from Thai markets seemed to parallel this acute disease both seasonally and geographically. In addition, attention was drawn to the marked similarities between acute aflatoxin B<sub>1</sub> poisoning in young monkeys and EFDV in Thai children and it was suggested that aflatoxins and possibly other mycotoxins may be a factor in the etiology of EFDV.

Molds may be present incidentally on many foods or, in more limited instances, as intentional ingredients of the final product. A large number of different kinds of molds, including *A. flavus* and many other aspergilli were found on the surfaces of country cured hams and "fermented" sausages.

A wide variety of commodities have been shown to yield aflatoxin in the presence of suitable strains of *A. flavus*; *e.g.*, barley, bean, black pepper-corns, brazil nut, cassava, cocoa bean, cocoa product, coconut oil cake, copra,



corn, corn grits, cottonseed meal, cowpea, hams (aged), italian type sausages, locust bean, millet, palm kernel, pea, peanut, peanut butter, peanut meal, pecan, raisin, rice, sesame, sorghum, soybean meal, wheat, and wheat flour.

*Ochratoxin*. *Aspergillus ochraceus*, another toxigenic organism within the genus, is also widely distributed in nature and its toxic principle has been termed ochratoxin. At least two other members of the *A. ochraceus* group, *A. sulphureus* and *A. melleus* (LAI *et al.*, 1968) and a penicillium, *P. viridicatum* have been found to elaborate ochratoxin (VAN WALBEEK *et al.*, 1969).

Early workers reported that ochratoxin had about the same order of oral toxicity as did aflatoxin B<sub>1</sub>, i.e. an LD<sub>50</sub> of 25 µg/duckling (VAN DER MERWE *et al.*, 1965) but this has been revised to about 150 µg/duckling (PURCHASE & NEL, 1967).

Several strains of *A. ochraceus* have been isolated from Brazil nuts, stored cottonseed, citrus fruit, hops, peanuts, South African stock feeds, and from tobacco. CHRISTENSEN *et al.* (1967) found that *A. ochraceus* and *A. flavus* were the predominant fungi of red pepper. A *Penicillium* species which produced ochratoxin A was isolated by VAN WALBEEK *et al.* (1968) from a surface growth of packaged hams.

In the Far East, *A. ochraceus* and related species were reported as constituents characteristic of the microflora of the fermented fish preparations, *katsubushi* and *katsuoshuto* [YUKAWA (1911) and KINOSITA *et al.* (1968) see STEYN (1971)]. In the U.S., Patent No. 1,313,209 covers the use of *A. ochraceus* for inducing a desirable change during the fermentation of coffee.

A strain of *A. ochraceus* isolated from moldy country-cured hams was able to produce ochratoxin A and B on rice and on peanut meal (ESCHER, 1971). On rice, 6 µg/g ochratoxin A and 15 µg/g ochratoxin B were found; on peanut meal, yields were 1 µg/g ochratoxin A and 4 µg/g ochratoxin B. Whether a toxin producing strain of *A. ochraceus* is also able to produce ochratoxins on ham itself has not been investigated.

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## EFFECT OF BRINE COMPOSITION ON THE FERMENTATION OF CUCUMBERS\*

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Various modifications of the traditional curing method used in Hungary have been tested. The effects of brine salinity, amount of added acetic and lactic acid, addition of alum or sugar and of preservatives, benzoic acid or sorbic acid, upon the fermentation of cucumbers were determined by chemical and microbiological analyses of the brine during the curing process. In addition, the texture and sensory properties of fermented cucumbers were evaluated with respect to brine composition.

Decrease in brine salinity resulted in an increased rate of bacterial growth and of their acid production but did not otherwise significantly affect the final outcome of fermentation. However, the taste of cucumbers fermented in lower salinity brine proved to be less satisfactory.

The addition of sugar resulted in a slight increase in the rate of acid production which did not, however, coincide with the population of acid-forming bacteria. Also there was an appreciable increase in the growth rate and in the final acidity as compared to the control but not in dependence of sugar concentration applied.

The lowest amount of acid of either kind added at start delayed the acid formation. This effect of lactic acid was more pronounced. When acetic acid was added the initial pH of the brine was higher than that of a respective brine of the same total acidity adjusted with lactic acid. Contrary to their retarding effect upon the fermentation, the acids added did not significantly influence the quantity of total acid produced. Lactic acid markedly inhibited the growth and activity of lactic acid bacteria and caused, on the other hand, an increased bloating of cucumbers. From the organoleptic point of view, however, the cucumbers fermented in lactic acid containing brine were considered the best by taste panels.

Alum had no significant effect upon any parameters of fermentation, even the texture of cucumbers remained unaffected.

Sorbic acid proved to be superior to benzoic acid. The former allowed, the latter decreased the growth and the activity of lactic acid bacteria. Sorbic acid on the other hand inhibited yeasts selectively.

In the manufacturing of cucumber pickles the activity of lactic acid bacteria is exploited by man. These bacteria normally occur in plant materials and cause their spontaneous fermentation. This spontaneous lactic acid fermentation is promoted and controlled by the use of brine.

The composition of the latter is, however, based on empirical experience and little systematical study has been made of the effect on the fermentation

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of the components of the brine. As brine composition is of importance also from the economical point of view, experiments were carried out to investigate the effect of brine components on the fermentation of cucumbers.

### 1. Methods

The experimental methods were published in detail earlier (DEÁK, 1963a, b) and will only briefly be treated here. The traditional brine used in Hungary for cucumber fermentation is composed of 5% salt, 0.35% acetic acid, 0.2% lactic acid and 0.1% alum. Several years ago benzoic acid was also involved as a preservative but has now mostly been substituted by sorbic acid for its favourable selective action. In the experiments each brine component was applied in 2 or 3 different concentrations, while sorbic acid or benzoic acid were used in 0.12% and 0.15% concentrations, respectively (Table 1). Each experiment was run in three parallels.

Table 1  
*The composition of experimental brines*

Treatment number	Salt %	Acetic acid %	Lactic acid %	Alum %	Sorbic acid %	Benzoic acid %	Invert sugar %
1	5	—	—	0.1	0.12	—	—
2	5	—	0.20	0.1	0.12	—	—
3	5	—	0.70	0.1	0.12	—	—
4	5	0.35	0.20	0.1	0.12	—	—
5	5	0.35	—	0.1	0.12	—	—
6	5	0.47	—	0.1	0.12	—	—
7	5	0.35	—	—	0.12	—	—
8	5	0.35	0.20	—	0.12	—	—
9	3	0.35	0.20	0.1	0.12	—	—
10	3	0.47	—	0.1	0.12	—	—
11	3	0.35	—	0.1	0.12	—	—
12	5	0.35	—	0.1	—	0.15	—
13	5	0.47	—	0.1	—	0.15	—
14	5	0.35	0.20	0.1	—	0.15	—
15	5	0.35	0.20	0.1	—	0.15	0.5
16	5	0.35	0.20	0.1	—	0.15	1.0
17	5	0.35	0.20	0.1	—	0.15	1.5

The experimental fermentations were performed in small barrels filled with 40 kg of cucumbers and 40 l of brine. The concentration of brine constituents, therefore, decreased by half after equilibrium had taken place. For chemical and bacteriological analyses, samples were taken at regular intervals through plastic tubes placed in the centre of the barrels. The acidity was determined by titration and expressed as lactic acid. The pH of the brine was determined by means of a glass electrode. For microbiological analyses the samples were examined by the plating technique using media selective for lactic acid bacteria and yeasts, resp. At the end of the experiments the cucumbers were weighed again to calculate the fermentation loss, then were sorted and subjected to taste panels for sensory evaluation.

## 2. Results

Typical curves of chemical changes occurring during fermentation are shown in Fig. 1. It is evident that the changes during the first days are due to the equalization by diffusion between the brine and cucumbers. The brine salinity and acidity decreased and a corresponding increase in pH appeared. The sugar curve indicates a rapid increase in the brine sugar concentration as a result of sugar diffusion from cucumbers. After an active fermentation had started the brine sugar began to decrease with a corresponding increase in acid content and a decrease in pH. Acid development continued until about the 18th day and pH decreased as long as acid was formed. Brine sugar practically disappeared by about the 20th day.

Average changes in microbial population are shown in Fig. 2. Rapid growth of acid-forming bacteria started immediately as the brine sugar

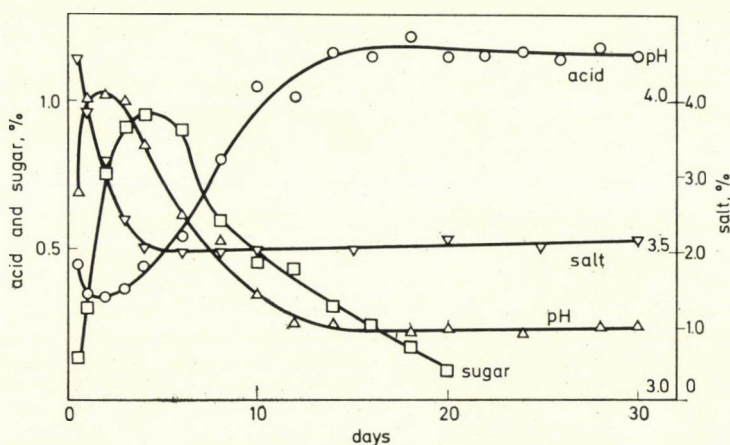


Fig. 1. Average chemical changes in cucumber fermentation



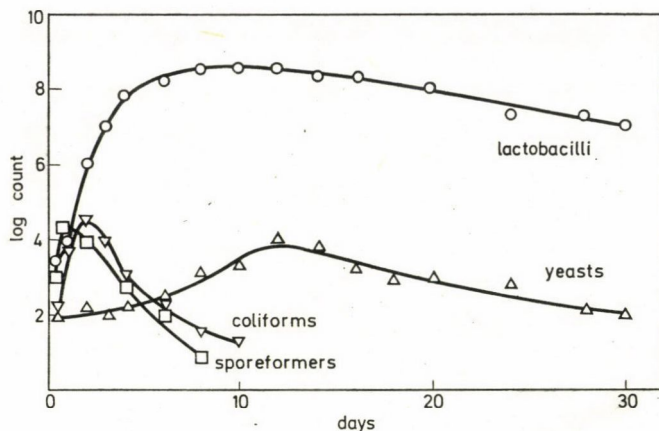


Fig. 2. Average microbiological changes in cucumber fermentation

became available. The growth curve of acid-forming bacteria reached a peak of more than 100 millions per ml on the 9th day and then slowly subsided. The first 4 to 5 days were characterized by a sharp drop in the population of aerobic sporeformers and coliform bacteria. The yeast population increased slowly, as the acidity of the brine was changing to more and more favourable conditions. Their number reached a peak on the 12th day and remained over several thousands per ml till the end. Note the great difference in the number of the yeasts and lactic acid bacteria.

These general chemical and microbiological features were markedly influenced by the treatments with various brine compositions. These influences will be reported in the following. For the sake of brevity, out of the experimental results only the acid production and the number of lactic acid bacteria will be summarized here as these two are the most characteristic of the fermentation. In addition, some other results will also be mentioned where of interest.

### 2.1. Acid production

In terms of acid produced the fermentation process may be characterized by three variables, *i.e.* the lag in acid production, the rate of acid production and the total amount of acid produced. However, to compare the various treatments in which different amounts of acids were added, the initial acid content of the brine should be subtracted. Taking measures in this way it is seen (Table 2) that any amount or kind of acid added at the start delayed significantly the fermentation. The lag was longer in each treatment receiving acid as compared to treatment No. 1. not containing any added acid. Among the acidic treatments, in turn, the highest amount of lactic acid differed significantly from the others.

Table 2  
*Effect of brine acidity on the acid production*

No.	Treatment		Lag, days	Rate, % per day	Total %
	Acetic acid %	Lactic acid %			
1	—	—	1.57	0.081	0.823
2	—	0.20	4.76	0.106	0.763
3	—	0.70	8.33	0.060	0.663
4	0.35	0.20	5.23	0.101	0.736
5	0.35	—	4.80	0.140	0.789
6	0.47	—	4.93	0.101	0.760
	LSD 5%		2.74	0.040	0.109

The rate of acid production was relatively lower in treatment No. 1, though differing significantly only from treatment No. 5 containing a lower amount of acetic acid. The rate of acid production was the highest in this treatment. In this respect, too, a higher amount of lactic acid lowered significantly the rate of acid production.

The retarding effect, on fermentation, of added lactic acid appeared in the total amount of acid formed, too, which, however, was significantly lower only as compared to treatments Nos. 1 and 5 while there was no significant difference between the others.

As to the effect of brine salinity (Table 3) little significant difference can be found in comparing the treatments of 3% and 5% salt. There were no

Table 3  
*Effect of salt and alum on the acid production*

No.	Treatment	Lag, days	Rate, % per day	Total %
4, 5, 6	Salt 5%	5.90	0.114	0.770
9, 10, 11	Salt 3%	4.99	0.137	0.762
	Difference	0.91	0.023	0.008
	$\pm s_d \cdot t_{5\%}$	1.21	0.018	0.139
4, 5	Alum 0.1%	4.73	0.124	0.730
7, 8	Alum 0%	4.97	0.120	0.795
	Difference	0.24	0.004	0.055
	$\pm s_d \cdot t_{5\%}$	0.96	0.023	0.088



significant differences in the lag and in the total amount of acid production either, while the rate of acid production was significantly higher in brine of 3% salt content.

Alum exerted no significant effect on either parameter of acid production (Table 3).

Addition of sugar to the brine caused no significant alteration at the start of the fermentation while it increased the total amount of acid formed. This increase was, however, independent of the amount of sugar added. On the other hand, the rate of acid production increased with sugar concentration (Table 4, Fig. 3).

Table 4  
*Effect of sugar on the acid production*

No.	Treatment	Lag, days	Rate, % per day	Total %
14	Sugar 0%	8.3	0.0541	0.702
15	Sugar 0.5%	7.8	0.0669	0.804
16	Sugar 1.0%	8.8	0.0723	0.820
17	Sugar 1.5%	8.7	0.0756	0.808
	LSD 5%	1.1	0.0063	0.060

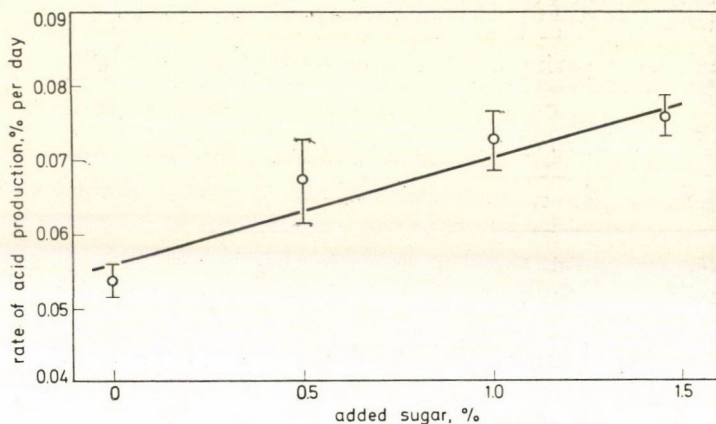


Fig. 3. Changes in the rate of acid production as a function of the amount of sugar added

Sorbic acid when used as preservative allowed the acid production to start earlier and to proceed at a higher rate than the respective benzoic acid treatments. However, this evidently more rapid fermentation did not result in a significantly higher amount of total acid production (Table 5).

Table 5  
*Effect of preservatives on the acid production*

No.	Treatment	Lag, days	Rate, % per day	Total %
4, 5, 6	Sorbic acid 0.12%	4.56	0.116	0.770
12, 13, 14	Benzoic acid 0.15%	6.38	0.082	0.742
	Difference	1.82	0.034	0.028
	$\pm s_d \cdot t_5\%$	1.23	0.023	0.082

## 2.2. Microbial population

In characterizing the effect of the various treatments on the microbial population the cell counts at start, *i.e.* on the 5th day, the growth rate constants, as well as the maximal and final counts will be considered.

In terms of the 5th day counts it is evident that the bacterial growth started much earlier in treatment without any added acid. Disregarding this extreme value other differences can be revealed which refer particularly to the inhibiting effect of lactic acid on the growth of acid forming bacteria (Table 6). More refined differences between the treatments can be found in comparing the growth rate constants. The growth rate was the highest in the treatment without acid and the lowest in the strong lactic acid treatment. 0.47% acetic acid decreased the growth rate significantly as compared to the effect of 0.35% acetic acid. The strong inhibiting effect of lactic acid is shown by the fact that 0.20% lactic acid affected the growth rate as strongly as 0.47% acetic acid.

Table 6  
*Effect of brine acidity on the population of lactic acid bacteria*

No.	Treatment		Cell count $\cdot 10^6$ per ml			Growth rate Generations per hour
	Acetic acid %	Lactic acid %	5th day	Maximal	Final	
1	—	—	13.560*	105.3	10.4	1.76
2	—	0.20	0.177	147.3	22.0	0.89
3	—	0.70	0.030	167.7	76.7	0.30
4	0.35	0.20	0.589	116.3	9.4	1.35
5	0.35	—	0.550	104.3	15.6	1.34
6	0.47	—	0.086	66.3	10.2	1.05
	LSD 5%		0.339*	51.0	18.8	0.18

\* The salient value of Treatment No. 1 was disregarded not to conceal the other differences.



The maximal and final counts point again to the inhibiting effect of lactic acid in that these counts were the highest in the lactic acid treatments as the fermentation has not yet finished in these due to the retardation caused by lactic acid addition.

The lower salt concentration of brine accelerated the outset of the bacterial growth as is shown by the higher values of the 5th day count and growth rate constant, but did not otherwise influence the maximal and final counts (Table 7).

Table 7

*Effect of salt, alum and sugar on the population of lactic acid bacteria*

No.	Treatment	Cell count $\cdot 10^6$ per ml			Growth rate Generations per hour
		5th day	Maximal	Final	
4, 5, 6	Salt 5%	0.41	95.6	11.7	1.25
9, 10, 11	Salt 3%	3.00	100.7	12.0	1.39
	Difference	2.59	5.1	0.3	0.14
	$\pm s_d \cdot t_5\%$	1.07	92.4	13.2	0.12
4, 5	Alum 0.1%	0.34	117.2	12.4	1.12
7, 8	Alum 0%	0.57	110.3	12.5	0.93
	Difference	0.23	6.9	0.1	0.19
	$\pm s_d \cdot t_5\%$	0.36	49.5	6.4	0.27
14	Sugar 0%	0.98	125.1	47.7	1.93
15	Sugar 0.5%	0.23	93.6	39.7	2.44
16	Sugar 1.0%	1.30	115.2	57.1	2.28
17	Sugar 1.5%	0.70	110.3	33.8	2.39
	LSD 5%	0.83	46.2	26.2	0.23

Alum had no effect on the bacterial population either. As to the addition of sugar the only significant effect can be found in the increase of the growth rate constant. However, this holds true only in comparison to the control as the increase of the growth rate constant was not proportional to the increase in sugar concentration.

Preservatives exerted very marked influences on the microbial population (Table 8). In the presence of sorbic acid the growth of lactic acid bacteria started earlier and proceeded quicker than in the presence of benzoic acid, as

is illustrated by the 5th day count and growth rate constant. In turn, the final count was higher in the benzoic acid treatments than in the sorbic acid ones that can be interpreted as the non-completion of fermentation.

Table 8

*Effect of preservatives on the population of lactic acid bacteria and yeasts*

No.	Treatment	Cell count · 10 <sup>6</sup> per ml			Growth rate Generations per hour
		5th day	Maximal	Final	
		Lactic acid bacteria			
4, 5, 6	Sorbic acid 0.12%	0.480	107.4	11.6	1.13
12, 13, 14	Benzoic acid 0.15%	0.124	90.0	55.8	0.82
	Difference	0.356	17.5	44.2	0.31
	± s <sub>d</sub> · t <sub>5</sub> %	0.322	42.5	55.8	0.20
		Yeasts			
4, 5, 6	Sorbic acid 0.12%	0.120	1.56	0.005	0.72
12, 13, 14	Benzoic acid 0.15%	0.254	25.00	0.594	2.30
	Difference	0.134	23.44	0.591	1.58
	± s <sub>d</sub> · t <sub>5</sub> %	0.116	16.28	0.154	1.03

Even more characteristic differences can be found between the effects of the two preservatives as to the yeast population. Yeast growth had started earlier and both rate of growth and maximal and final counts were significantly higher in benzoic acid treatments than in sorbic acid lots pointing to the strong selective inhibiting effect of sorbic acid on yeast.

### 2.3. Other results

Some additional data on differences in the quality of fermented cucumbers induced by various treatments are worth mentioning.

In general, the sensory properties of fermented cucumbers resulting from all the treatments proved to be acceptable as revealed by taste panel tests. Two findings are only to be mentioned. The first is that the taste of cucumbers of treatment No. 3 which contained a high amount of lactic acid and the parameters of which proved to be inferior in many respects was claimed to be the best as verified by the significant score number of the triangular test. The second finding concerns the taste of cucumbers fermented in brine containing



3% salt. In panel tests its samples were scored as being of significantly lower quality and the judgement was that they had some sort of an empty taste.

The data on the evaluation of the hardness and the bloating of cucumbers reveal that alum did not improve the hardness of cucumbers or cause any increase in the occurrence of bloaters. On the other hand, the softness and the bloating of cucumbers were significantly increased by lactic acid used in the lower concentration but not in the higher (Table 9).

Table 9

*Effect of brine acidity and alum on the hardness and the bloating of cucumbers*

No.	Treatment		Hard cucumbers %	Hollow cucumbers %
	Acetic acid %	Lactic acid %		
1	—	—	67.6	27.4
2	—	0.20	58.0	33.3
3	—	0.70	68.0	25.8
4—8*	0.35	0.20	69.7—63.1	24.1—29.8
5—7*	0.35	—	71.7—73.0	22.5—20.8
6	0.47	—	64.7	28.7
LSD 5%			8.7	9.3

\* Treatments Nos 7 and 8 without alum

### 3. Conclusions

On the basis of the experimental data presented the following conclusions can be drawn:

#### 3.1. The effect of salt

It is well established that the most important constituent of brine in controlling spontaneous acid fermentation is salt (JONES & ETCHELLS, 1943; ETCHELLS & JONES, 1943; FABIAN & BLUM, 1943). It causes the cells of the cucumber tissue to become permeable and forces the soluble cellular constituents to diffuse into the brine. Out of these constituents sugar is of the greatest importance as it is converted by bacteria to acids. Salt controls the activity of bacteria occurring naturally on the cucumbers and in the brine, in that it allows only the salt tolerant organisms as lactic acid bacteria and yeasts to utilize the sugar and other nutritive materials. In addition, salt contributes to the development of the desired taste of cucumber pickle.



So there is no question about the role and importance of salt as brine constituent and the only point aimed at here was to test whether a lower salt concentration than the one used traditionally in this country would be practicable or not. The results showed that although 3% salt in the brine would favour the outset of acid production by lactic acid bacteria — as evidenced by the increase in the 5th day counts, the rate of growth and of acid production — yet did not result in a higher total acid production, and, what of greater importance, the lower salinity brine gave an objectionable empty taste to the finished cucumbers.

### 3.2. *The effect of acids*

The addition of acids to the brine would serve the purpose to create more favourable conditions for the acid producing bacteria and, inversely, to create less favourable or even adverse conditions for the undesired microorganisms which would cause spoilage or impaired quality.

Out of the microorganisms constituting the heterogenous microflora at the start of the fermentation the sporeformers, *i.e.* the members of the genera *Bacillus* and *Clostridium* would potentially be most dangerous being capable of softening the cucumbers by producing pectin splitting enzymes (NORTJE & VAUGHN, 1953; VAUGHN *et al.*, 1954). These bacteria as well as the coliform bacteria, the latter causing gaseous fermentation and consequently bloating of cucumbers (ETCHELLS *et al.*, 1945) are, indeed, strongly inhibited by the acidification of the brine. Their number falls, to a negligible value in a few days, as it was demonstrated in Fig. 2.

It should be added that besides their low acid tolerance these bacteria cannot bear higher salt concentration either. Thus even the salt content of the brine retards their growth to some extent.

On the other hand, however, it is evident from the results presented that either acid in any concentration used in the brine inhibited also the growth and acid production of lactic acid bacteria. This inhibition was reflected in one or more of the parameters studied as compared to the control containing no added acid but appeared mostly in the lag and the rate of acid production and in the 5th day count of lactic acid bacteria and in their growth rate. In other words, the acids inhibited particularly the outset of the fermentation and decreased the rate of it. This means that those advantages of acid addition, which would arise from the selective action on the heterogenous flora of microorganisms at start, are mostly compensated by the inhibition, though to a lesser degree, of lactic acid bacteria themselves.

However, this delaying and retarding effect of acids does not appear in the final outcome of the fermentation as it has been indicated by the non-significant differences, as compared to the control, in the total amount of acid



produced and in the maximal and final counts of lactic acid bacteria. These conclusions are in agreement with the data of literature (JONES *et al.*, 1940).

There is a marked difference between the effects of the two acids tested. The inhibition caused by acetic acid was less pronounced than that caused by lactic acid. Addition of a 0.35% acetic acid alone resulted in a fermentation characterized by parameters differing but slightly from those of the control without added acid. In turn, lactic acid exerted a retarding effect on the fermentation and, in general, resulted in parameters significantly inferior to the control.

Lactic acid addition not only inhibited the growth and activity of lactic acid bacteria but it also impaired the texture of the fermented cucumbers, causing softening and bloating. Certainly, this effect cannot be directly related to lactic acid as it occurred in treatments with lower but not higher amounts of lactic acid. According to the literature, it is rather probable that the formation of hollows in, and the loss of hardness of, the cucumbers could be attributed to the activity of yeasts (ETCHELLS *et al.*, 1945; JONES *et al.*, 1941; VELDHUIS *et al.*, 1941) which might be allowed to act in the presence of lower amounts of lactic acid in the brine but might be retarded by the addition of a higher amount of lactic acid. The disadvantageous effect of lactic acid addition is not contradicted by the fact that in the sensory evaluation by taste panels of fermented cucumbers the samples from treatment receiving a higher amount of lactic acid were claimed to be the best. On the one hand all the other samples but the one representing the fermentation in lower salinity brine, proved to be satisfactory and acceptable. On the other hand, this result of the quality evaluation points to the fact that the trained members of the panels looked for and preferred particularly the characteristic taste of lactic acid of pickles which was undoubtedly most pronounced in the samples to which a high amount of lactic acid was added.

In conclusion, concerning the addition of acids to the brine it can be accepted if for securing the fermentation, storage and quality of cucumber pickles a small amount of acetic acid is added to the brine, particularly in the case of relatively low brine salinity as in the process practiced in this country. However, addition of lactic acid seems to be rather unadvisable.

### 3.3. The effect of sugar

There have been several suggestions as to the addition of sugar to the brine in order to accelerate acid formation or to favour the production of a higher amount of acid (JONES *et al.*, 1940). In this experiment the addition of sugar caused no significant change in parameters characterizing the beginning of the fermentation, *i.e.* it did not shorten the lag nor increase the 5th day counts of acid forming bacteria. It did, however, accelerate the rate of acid

production which was directly proportional to the sugar concentration applied. Increases in the growth rate of acid forming bacteria and in the final acidity were also observed as compared to the control. These increases, however, did not coincide with the increase in sugar concentration. These results cannot be considered unambiguous evidence for the advantage of sugar addition. Even if a distinct increase in brine acidity would be expected following the addition of sugar to the brine, its practicability from the economical point of view remains to be reconsidered. Moreover, there are reports in the literature indicating that the addition of sugar would bring about a large increase in the proportion of bloaters which seems to be correlated to the increase of the yeast population (VELDHUIS *et al.*, 1941; RAGHEB & FABIAN, 1957). It is felt that sugar addition to the brine is questionable and rather undesirable.

### 3.4. *The effect of alum*

Alum has been used in pickle production for many years and the manufacturers are unanimous in claiming that it crisps and firms the cucumbers. The few data available in the literature concerning the addition of alum to the brine are, however, not so uniform in evidencing the advantage attributed to the use of alum and there are at least two reports indicating that alum would retard the development of acid and promote the formation of hollow cucumbers (FABIAN & KRUM, 1949; SAMISH *et al.*, 1957).

From the results presented here it can be ascertained that alum had neither positive nor negative influence on the fermentation as there were found differences of no significance whatever with respect to the parameters studied upon the addition of alum to the brine. Alum did not even increase the percentage of hard and crisp cucumbers and thus failed to serve the only purpose it was intended for.

### 3.5. *The effect of preservatives*

Turning to the last point, it is easy to come to a conclusion on the basis of results of this series of experiments. The data presented have consistently shown the superiority of sorbic acid to benzoic acid as a preservative in pickle processing. Each of the parameters investigated can be interpreted as indicating that benzoic acid inhibited the course of fermentation while sorbic acid did not. On the other hand, benzoic acid allowed while sorbic acid inhibited the growth of yeasts. It is this selective inhibitory action of sorbic acid which makes it particularly useful in pickling (PHILLIPS & MUNDT, 1950; COSTILOW, 1957; DEÁK, 1959, 1960).

As mentioned before, out of the members of the heterogenous initial microflora, the sporeformers and the coliforms are inhibited by the addition



of salt, particularly if the brine contains some acid. In these circumstances it is the yeasts which form the only group of microorganisms showing a degree of salt and acid tolerance comparable at least to that of lactic acid bacteria. Yeasts are thus capable of competing with the latter for the sugar and other nutrients. Under conditions of cucumber fermentation two types of yeast may be active and both cause faults. These are the fermenting yeasts and the film-forming yeasts (ETCHELLS & BELL, 1950a, b). The former bringing about gaseous fermentation could be associated with bloater production. The latter may form heavy film or scum on the surface of the fermenting tanks. This is a sign of their oxidative metabolism by which they are enabled to assimilate the acidic products of fermentation and by which, in turn, they might prepare the way for spoilage and impair the keeping quality of the product. Softening is sometimes ascribed also to film-forming yeasts. Now, all these hazardous activities of yeast can successfully be prevented by the use of sorbic acid (DEÁK, 1962).

It is beyond the scope of this lecture to cover in more detail the usefulness of sorbic acid as preservative and just keeping to the point it will be sufficient to say, based on the experimental evidence presented, that if one intends to ensure satisfactory curing and subsequent storage of cucumbers by applying a preservative, it is certainly advisable to choose sorbic acid to meet this purpose.

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## TECHNOLOGICAL QUALITY OF MINCED FISH PRESERVED BY FREEZING AND ADDITIVES\*

J. GRABOWSKA and Z. SIKORSKI

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The effect of exhaustive water extraction and/or addition of sucrose, molasses, sodium monoglutamate, sodium chloride, polyphosphates, and wood smoke condensate to minced fish before freezing and storage on the suitability of the minces as components of sausage emulsions was investigated. Frozen storage resulted in rapid decrease of protein solubility in the thawed material and deterioration of the texture of the sausages. Minced fish obtained from cod fillets was superior to that made from gutted fish in a skin and bone separator. Water extraction and addition of glucose efficiently improved the technological value of the minced fillets. Glucose was more effective when applied to water-extracted material. Other additives did not significantly retard undesirable protein and texture changes.

In the recent few years a significant decrease in the availability of many commercially exploited fish species was noted in several traditional Atlantic fishing grounds (Fig. 1). In order to supply the necessary amounts of animal protein for human nutrition attempts are made to utilize several abundant, less valuable species, previously not fished on industrial scale (Fig. 2). However, because of the different morphometric characteristic of these fish and/or their less attractive sensory properties new ways of utilization of their edible parts are searched for. According to one of the industrial procedures the fish meat is machine separated on board ship, extracted with water and preserved by freezing to be later used for the production of fish sausages, fish patties or various jelly-like products.

The technological value of the machine-separated, minced tissues, especially the suitability for sausage emulsions deteriorates during frozen storage more rapidly than that of fillets or round fish. Thus the raw material has to be preserved by combined action of freezing and additional treatment. According to Japanese workers (IKEUCHI & SIMIZU, 1963; OKADA, 1964, 1969) the storage life of frozen minced fish may be significantly extended by water extraction prior to freezing and the use of various additives, like sucrose, glucose, polyphosphates, sodium citrate. The deterioration of minced fish meat during frozen storage is manifested by protein denaturation. In sausage formulations

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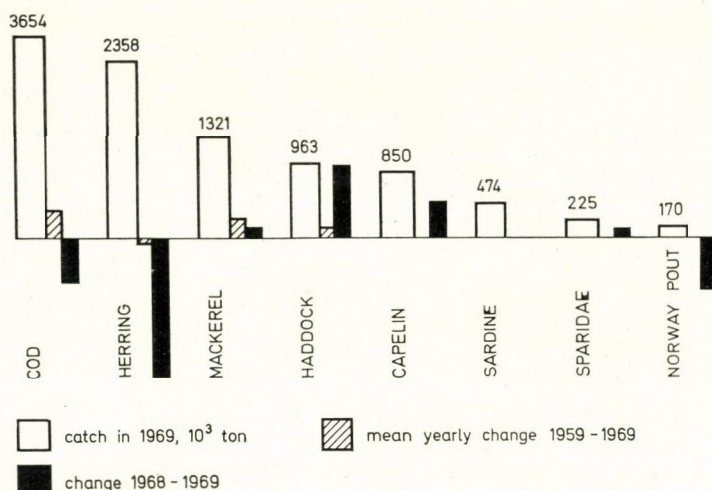


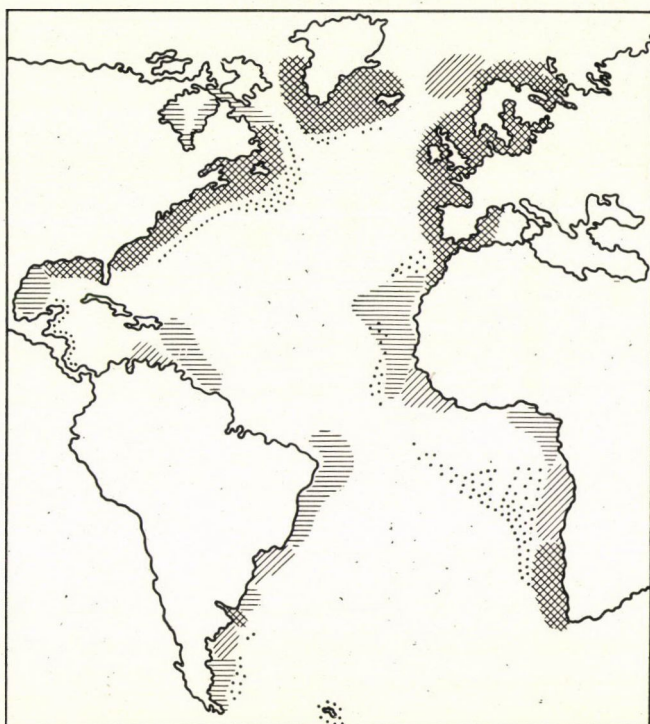
Fig. 1. Changes of catches of Atlantic fish species

such stale raw material induces undesirable grainy texture of the final product. It was attempted to evaluate the suitability of minced fish — preserved by combined action of freezing and additives, not extracted with water before freezing — for sausage emulsions on the basis of its protein solubility.

### 1. Materials and methods

Minced edible parts obtained from fresh gutted Baltic cod (*Gadus callarias*) in a skin and bone separator Farsh-2 (Fig. 3) or from cod fillets in a silent cutter, were mixed with additives, frozen and stored at  $-18^{\circ}\text{C}$  for different periods of time (Fig. 4). The following additives were used: sucrose 0.5–10%, molasses 5%, sodium monoglutamate 2%, sodium chloride 0.6–5% commercial mixture of polyphosphates Polital M 5a 0.2–0.5%, and wood smoke condensate (Polish Meat Institute) 10 ppm, separately and in combinations. The mince was used after thawing to prepare comminuted fish sausages of the frankfurter type. The composition of the sausage formulations was kept constant (SADOWSKA & SIKORSKI, 1972; SIKORSKI *et al.*, 1972).

The minced meat was characterized before freezing and after frozen storage by determining all salt soluble proteins according to SAFFLE and GALBREATH (1964) and the myofibrillar proteins by the procedure of DYER and SNOW (1950). In model experiments myofibrillar proteins in solutions were treated with additives, after freezing and frozen storage the insoluble fraction was separated by centrifugation and protein concentration was deter-







-  intensively exploited areas
-  fisheries possible to expand
-  little exploited areas
-  unexploited resources

Fig. 2. Exploitation of Atlantic fishery resources

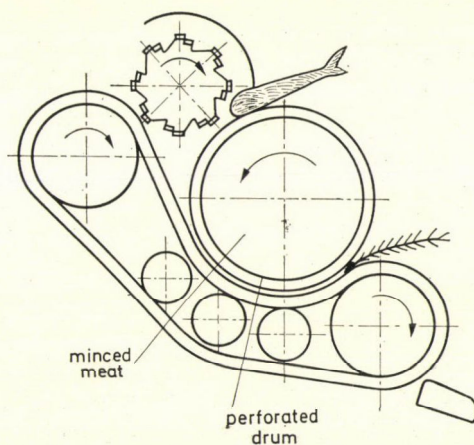


Fig. 3. Skin and bone separator FARSH-2



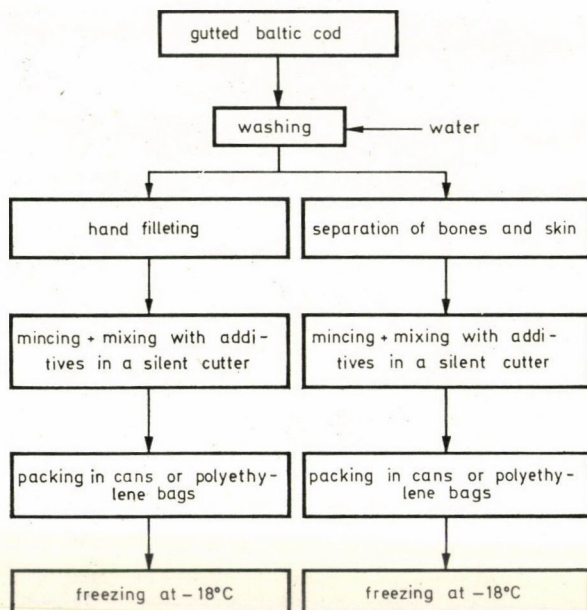


Fig. 4. Preparation of minced fish meat

mined in the supernatant. According to SNOW (1950) and to NOGUCHI and MATSUMOTO (1970) solubility changes induced by freezing and storage in protein solutions are correlated to those in whole tissues.

## 2. Results

Machine-separated edible parts of Baltic cod when used in fresh state for sausage emulsions yielded an acceptable texture of the final product. However, when frozen after additional mixing in a commercial silent cutter without refrigeration deteriorated rapidly during storage and were not acceptable for use in sausage formulations because of unappealing grainy texture and the separation of water and fat. This was accompanied by a rapid decrease of protein solubility when determined in the thawed mince, even below the level of sarcoplasmic protein concentration. Addition of sucrose, molasses, sodium monoglutamate, sodium chloride, polyphosphates, and wood smoke condensate, separately and in combinations, did not improve the technological fitness nor the protein solubility in the fresh and frozen material (Table 1).

Minced meat obtained from cod fillets in a silent cutter retained its suitability as raw material for sausage emulsions up to 6 weeks of frozen storage at  $-18^{\circ}\text{C}$  (Table 2). Addition of sucrose improved the protein solubility (Figs. 5, 6).

Table 1

*Effect of additives on protein solubility in frozen minced fish prepared in the FARSH-2 separator*

Sample	Storage (weeks)			
	2		16	
	Protein solubility*	Texture of the fish sausages**	Protein solubility*	Texture of the fish sausages
<i>Controls:</i>				
Unfrozen minced meat	66–70	+		
Frozen minced meat	22–24	+ –	9–11	
<i>Frozen, with additives</i>				
2% Na-glutamate	33–35	+ –	7–8	Unacceptable
5% molasses	33–35	+ –	11–12	
5% molasses and 0.2% poly-phosphate	20–22	–	8–10	
1% sucrose and 2.5% NaCl	24–26	–	6–7	
3% sucrose and 2.5% NaCl	33–35	–	8–10	
5% sucrose and 2.5% NaCl	24–27	–	8–10	
2.5% salt	10–15	–	4–6	
10 ppm smoke condensate	24–26	–	10–12	
0.2% polyphosphate	33–35	+ –	8–10	
0.5% polyphosphate	33–35	+ –	8–10	

\* Per cent of value found in fresh fillet

\*\* + Acceptable

+ – Grainy, evident loss of gelled structure

– Unacceptable, fat and water separated

Table 2

*The influence of storage time on protein solubility of frozen minced cod fillets and on texture of fish sausages*

Storage time (weeks) –18 °C	Whole minced fillets		Extracted with water		Extracted, 2% NaCl add.	
	Texture* g/cm <sup>2</sup>	Protein solubility**	Texture* g/cm <sup>2</sup>	Protein solubility**	Texture* g/cm <sup>2</sup>	Protein solubility**
0	540 ± 27	85	645 ± 32	85	547 ± 27	70
3	676 ± 34	80	676 ± 34	80	364 ± 18.2	45
6	523 ± 26	65	556 ± 28	80	320 ± 16	40

\* cited from SIKORSKI *et al.* (1972); critical value 400 g/cm<sup>2</sup>

\*\* % of original concentration in fresh fish

No changes in protein solubility were observed after 12 weeks of frozen storage if the water-soluble fractions had been removed prior to freezing and 10% of glucose had been added. In water-extracted control samples the solubility of myofibrillar proteins decreased reaching about 40% of that in fresh meat (Fig. 7).



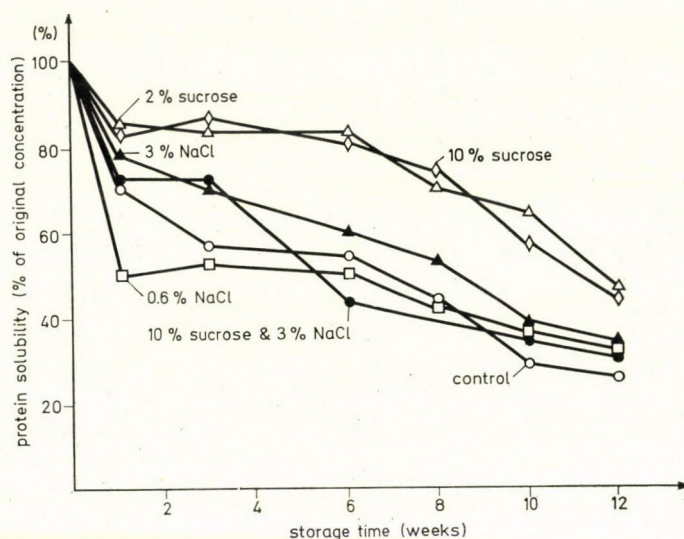


Fig. 5. The influence of additives on the solubility of proteins in frozen stored minced meat from cod fillets

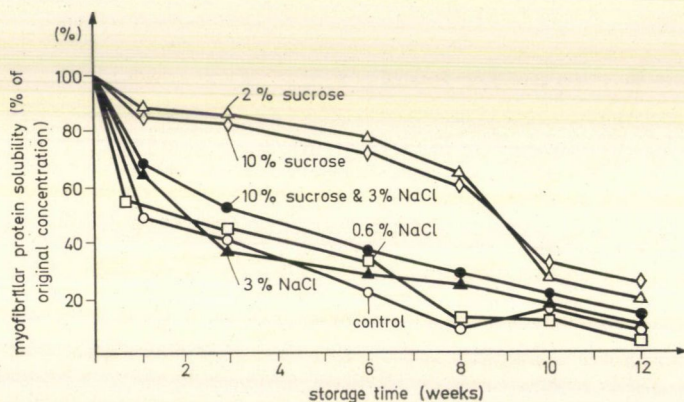


Fig. 6. The influence of additives on the solubility of myofibrillar proteins in frozen stored minced meat from cod fillets

In model experiments, where water-extracted minced fish meat was represented by myofibrillar protein solutions, glucose (2–10%) very efficiently inhibited the denaturation in stored samples (Fig. 8).

Other additives did not significantly influence the protein solubility in model solutions.

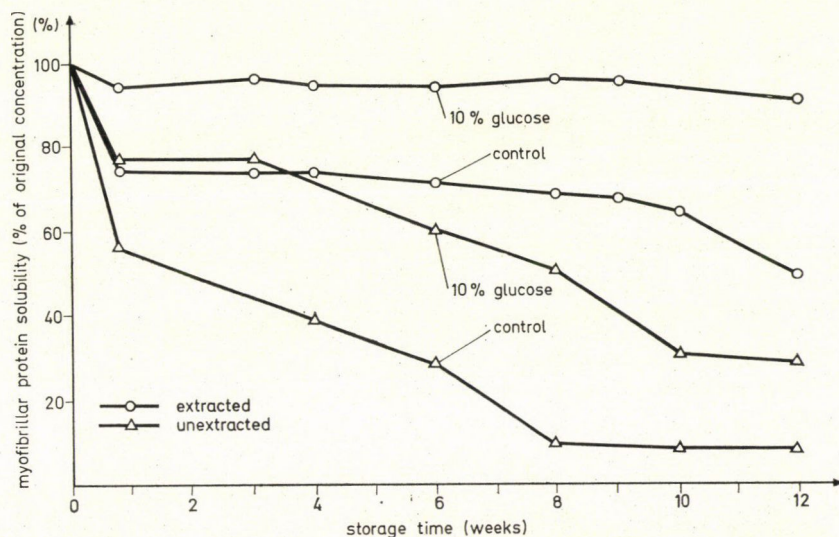


Fig. 7. The effect of glucose on protein solubility in whole and extracted minced cod fillets

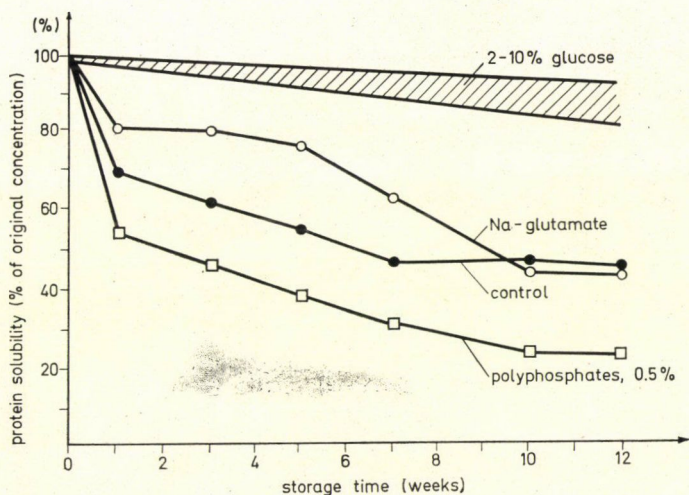


Fig. 8. Solubility of myofibrillar proteins frozen with additives

### 3. Conclusions

Minced fish obtained under industrial conditions in a skin and bone separator, not extracted with water, was, after frozen storage, not suitable as a component of sausage emulsions. The different additives tested did not improve its technological value. Considering the positive results obtained with unextracted minced cod fillets it seems reasonable to explain the difference



by the presence of a larger proportion of hematin compounds and other soluble components, originating in the inedible parts of the fish.

Addition of glucose is more effective in extending the storage life of pure minced frozen cod meat when applied after exhaustive water extraction.

The texture and binding of comminuted fish sausages was found to follow a pattern similar to solubility changes of myofibrillar proteins in the minced frozen fish. Thus factors inhibiting protein denaturation in frozen fish mince improve its technological suitability.

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## ENHANCING EFFECT OF CHEMICALS ON THE THERMAL INJURY OF MICROORGANISMS\*

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The enhancing effects of sodium chloride, tylosin and sorbic acid on the thermal destruction of *Candida utilis* and *Escherichia coli* were investigated. Increased sensitivity to sodium chloride and other solutes and leakage of intracellular substances could be observed in the heat-treated cells.

Although the amount of leakage is not proportional to the loss in viability of cells, the damage of cell membranes was proved by the decrease of solute tolerance and the increased sensitivity to tylosin. The enhancing effect of sodium chloride upon the thermal destruction of *C. utilis* and *E. coli* was observed mainly during the recovery period after heating while, on the contrary, sodium chloride acted protectively during heating. It is indicated that sorbic acid and tylosin have a greater effect on the inhibition of cellular repair mechanism after heating, and the enhancing effect of sorbic acid against *C. utilis* is due to the inhibition of the protein synthesis and respiratory activity during the repair process of heat-injured cells.

Many factors have been found to influence the lethal effects on microorganisms of temperatures elevated above those suitable for growth (RUSSELL, 1971; SHIBASAKI, 1969b; SHIBASAKI, 1971).

The possibility of reducing the heat resistance of microorganisms by chemicals has been investigated for a number of years. It has been found that various chemicals such as food preservatives, disinfectants, antibiotics have an enhancing effect on the lethal action of heat. This is an interesting problem for the evaluation of the mechanism of the lethal action of heat and for the purpose of increasing the efficiency of food preservatives and improving the food quality of heat-processed foods.

Although the combined effect of chemicals has been reported by many workers, the mechanism of, and the relationship between, chemical structure and antimicrobial characteristics against the enhancing effect of chemicals had not yet been investigated in detail.

The knowledge recently obtained on the thermal injury of microorganisms is useful for the evaluation of the combined effect of chemicals on the thermal destruction of microorganisms. There are a series of studies on the thermal injuries and their recovery concerning *Staphylococcus aureus*

\* Presented at the IUFOST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



(IANDOLO & ORDAL, 1966; ALLWOOD & RUSSELL, 1968, 1969), *Streptococcus faecalis* (CLARK *et al.*, 1968), *Escherichia coli* (RUSSELL & HARRIES, 1967), *Salmonella typhimurium* (CLARK & ORDAL, 1969; DIERSON *et al.*, 1971; TOMLINS & ORDAL, 1971), *Aerobacter aerogenes* (STRANGE & SHON, 1964), and *Penicillium expansum* (BALDY *et al.*, 1970).

The thermal injury of microorganisms by mild heating has been characterized by the loss of cellular membrane integrity, as demonstrated by the leakage of amino acids, 260 nm absorbing substances and potassium ion, and increased sensitivity to sodium chloride, the degradation of ribosomal ribonucleic acid and some metabolic damage.

The authors have investigated the antimicrobial characteristics and the practical uses of several food preservatives since 1950.

In a series of these studies, the combined effect of chemicals, and one or more chemicals plus other means were also tested (SHIBASAKI & HORIE, 1968; AMANO *et al.*, 1968). The combined effects of mild heating and chemicals were tested using sorbic acid, dehydroacetic acid, benzoic acid and alkyl-p-hydroxybenzoates against several yeasts (SHIBASAKI & IIDA, 1968; SHIBASAKI, 1969a), and furylfuramide, tylosin (SHIBASAKI *et al.*, 1966), glycine, glycylglycine, ethylene diamine tetraacetic acid and polyphosphates against *Bacillus* spores (SHIBASAKI, 1970).

The aim of this paper is to describe the reduction of the thermal resistance of *Candida utilis* and *E. coli* (*Pseudomonas aeruginosa*) by the addition of sodium chloride, tylosin, and sorbic acid during the heating and recovery period, and the environmental factors as well as the mechanism of their enhancing effects on thermal injury of microorganisms.

## 1. Materials and methods

### 1. Chemicals and microorganisms employed

The chemicals employed in this experiment were sorbic acid (as potassium salt), furylfuramide (supplied by Ueno Fine Chemical Ind., Ltd.), tylosin (as lactate, supplied by Shionogi and Co., Ltd.), and other chemical reagents, obtained from other commercial sources.

All the test organisms used were strains obtained from the culture collection of the Department of Fermentation Technology, Osaka University.

*C. utilis* was cultured in Czapek-Dox's medium containing polypeptone and yeast extract at a concentration of 0.25% each (CDPY). Twenty ml of this medium was placed in a 100 ml Meyer flask, autoclaved at 121 °C for 10 min., after cooling a loopful of 2-day-old culture of the test organism was inoculated, and incubated under shaking at 30 °C for 20 or 40 hours.



The cells were harvested by centrifuge, washed twice in physiological saline, and suspended in the same saline.

*E. coli* (*Ps. aeruginosa*) was cultured under shaking in a nutrient broth (meat extract 0.5%, polypeptone 1.0%, yeast extract 0.5%, NaCl 0.5%, adjusted to pH 7.0) at 37 °C for about 20 hours. After the subculture had been performed twice under the same conditions, the cells were collected by centrifuge, washed twice in M/10 phosphate buffer (pH 7.0) and suspended in the same buffer.

### 1.2. Conditions of heat treatment

The cells of *C. utilis* were heated at various temperatures in a suspending menstruum of the following composition: 98 ml of M/10 phosphate buffer (pH 4.0) + 1 ml of a chemical solution + 1 ml of a cell suspension.

The samples were taken periodically and the number of viable cells was counted on CDPY agar medium at 30 °C for 2 to 4 days.

The degree of thermal injury of *C. utilis* was determined by the following procedure; 20 ml of fresh medium was inoculated by the addition of the subculture suspension at a concentration of 10%, and incubated at 30 °C under shaking. When the value of optical density reached about 2.0, the culture medium was heated for a constant period, cooled to 30 °C, and allowed to continue to incubate.

The time course of growth was determined by the measurement of optical density at 660 nm. Viable cells were calculated from the average number of colonies formed on CDPY agar medium at 30 °C for 2 or 5 days. The cells of *E. coli* were heated in M/10 phosphate buffer at pH 7.0, sampled periodically and the viable cells were plate-counted on a nutrient agar at 37 °C for 2 or 4 days.

The inhibition of the growth of the heated cells was observed with the same procedure as in the case of *C. utilis*, except using a nutrient broth as a medium.

### 1.3. Leakage of intracellular substances

The amount of leakage of intracellular substances from the heat-treated cells was estimated by the following method.

Five-ml samples of heated suspension were taken at intervals, centrifuged at 3 000 rpm for 15 min and the optical density at 260 nm of the supernatant fluid was estimated with a spectrophotometer (Hitachi model 101).



#### 1.4. Recovery of thermally injured cells

In an experiment as to the recovery of thermally injured cells of *C. utilis*, 2 ml of 16-hr cultured cells were added to 20 ml of a fresh medium and incubated at 30 °C under shaking. When the value of optical density at 660 nm of the cultured broth reached 2 or 3, the culture broth was heat-treated, or the cells were collected by centrifuge, suspended in M/10 phosphate buffer, and heated at a proper temperature. After the heat treatment, the cells were incubated under suitable conditions. Czapek-Dox's medium, phosphate buffer and so on were used as incubating media. Samples were taken out periodically and the viable cells were plate-counted on CDPY medium or CDPY medium + NaCl at 30 °C for 4 or 5 days.

The effect of inhibitors on the recovery from thermal injury was evaluated by the addition of a proper concentration of inhibitor to an incubating medium or plate-counting medium.

The same procedure as with *C. utilis* was applied for examining the recovery of the thermally injured cells of *E. coli*, except using a nutrient broth as a medium and the cell suspension harvested in the stationary phase.

#### 1.5. Biosynthesis and fractionation of intracellular substances

The time course of the biosynthesis of intracellular substances in the thermally injured cells of *C. utilis* was determined by the incorporation of  $^{14}\text{C}$ -adenine (2  $\mu\text{Ci/culture}$ ) or  $^{14}\text{C}$ -*Chlorella* amino acids (4  $\mu\text{Ci/culture}$ ) into the fraction of nucleic acid or protein. Czapek-Dox's medium containing 0.1% yeast extract was used in an experiment of the incorporation of  $^{14}\text{C}$ -adenine and 0.4% casamino acid was added to Czapek-Dox's medium in a case of  $^{14}\text{C}$ -amino acids. RNA, DNA and protein in the treated cells were fractionated by the Schmidt—Thanhauser—Schneider method. Three ml of ethanol was added to 0.2 ml of sample fractionated, and the radioactivity was counted by a liquid scintillation counter (Nuclear Chicago Co., type 6301). The respiratory activity of the thermally injured cells was measured with Warburg's manometer.

## 2. Results

#### 2.1. Thermal injury of microorganisms

It is desirable to elucidate the thermal injury of microorganisms employed in this investigation for the study of the mechanism of the combined effect of heat and chemicals.

The relationship between the viability and the amount of leakage from the cells of *C. utilis* and *E. coli* was evaluated under the conditions of exposure to temperatures ranging from 45° to 60 °C in M/10 buffer at a proper pH. The results obtained are shown in Fig. 1 and Table 1.

Although the amount of substances absorbing at 260 nm released from heated cells increased with increasing the heating temperature, it was not proportional to the loss in viability of cells. It was further shown by the authors (TSUCHIDO *et al.*, 1972a) that the rates of leakage from heated cells

Table 1

*Relationship between the cell viability and leakage of 260 nm absorbing substances*

Microorganism	Heating conditions	Reduction in cell viability	Absorbance at 260 nm
<i>C. utilis</i>	50 °C, 20 min, pH 4.0	$10^{-2}$	0.10
	55 °C, 10 min, pH 4.0	$10^{-4}$	0.10
	60 °C, 5 min, pH 4.0	$10^{-5}$	0.50
<i>E. coli</i>	50 °C, 120 min, pH 7.0	$10^{-2}$	0.10
	55 °C, 70 min, pH 7.0	$2 \times 10^{-4}$	0.08
	60 °C, 10 min, pH 7.0	$10^{-5}$	0.09

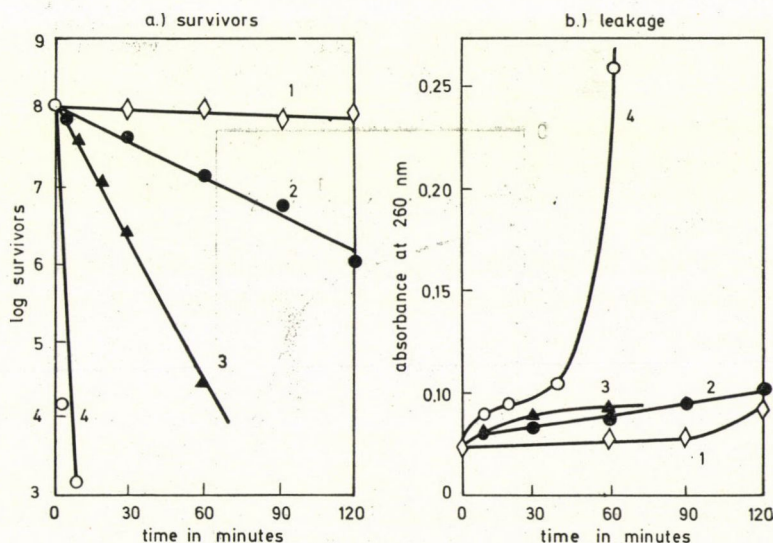


Fig. 1. Effect of temperature on the viability (a) and leakage of 260 nm absorbing substances (b) from the cells of *E. coli*. The cells were heated in M/10 phosphate buffer at pH 7.0, plate-counted on a nutrient agar at 37 °C for 2 days. 1, 2, 3, 4: heated at 45°, 50°, 55° and 60 °C, respectively



were low in the initial heating period in which the viability was rapidly lost, and thereafter the rates became significantly higher.

From these results it may be concluded that the leakage of the intracellular substances seems to be the secondary phenomenon in the heat injured cells, although the damage of cell membrane by the thermal stress was proved by another experiment to be described later.

Figs. 2, 3 and 4 indicate the effect of the addition of sodium chloride to the recovery medium on the survivors of *C. utilis* and *E. coli*. Ninety

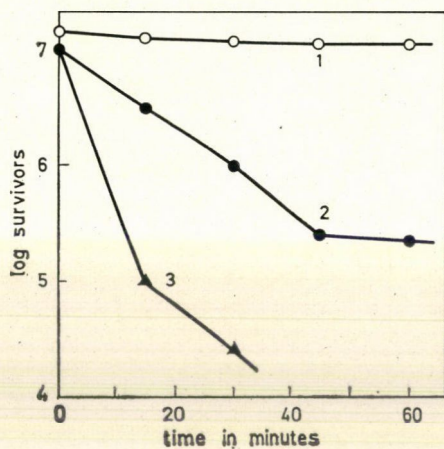


Fig. 2. Effect of NaCl in the recovery medium on the survivors of *C. utilis*. The cells were heated at 45 °C in M/10 phosphate buffer at pH 4.0. 1: plate-counted on CDPY agar. 2, 3: plate-counted on CDPY agar plus 7% NaCl, used cells were in the stationary phase or the log phase, respectively

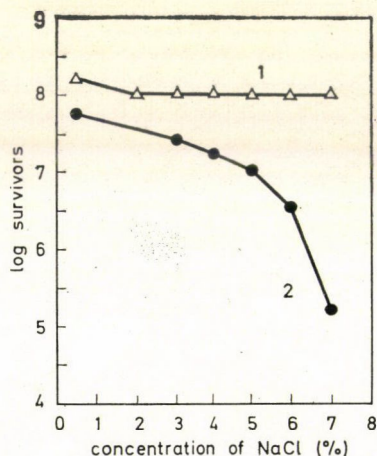


Fig. 3. Effect of NaCl in the recovery medium on the survivors of *E. coli*. The cells were heated at 55 °C for 3 min in M/10 phosphate buffer at pH 7.0, plate-counted on a nutrient agar plus NaCl. 1: unheated; 2: heated

per cent or more cells of these microorganisms became more sensitive to the relatively high concentration of NaCl in the recovery medium by mild heating at 45 °C for 20 to 60 min (*C. utilis*) and at 55 °C for 3 to 8 min (*E. coli*). The effect of the high concentration of other solutes such as KCl or sucrose is indicated in Fig. 5. The heat-treated cells of *E. coli* became more sensitive to KCl or sucrose similar to NaCl, but did not become more sensitive to glycerol (0.2 to 1.4 M).

When the enhancing effect of chemicals on the thermal destruction of microorganisms was evaluated by means of viable cell counts and the degree

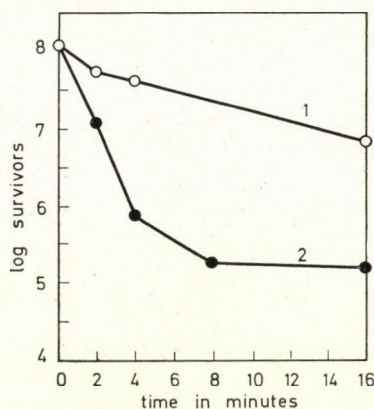


Fig. 4. Effect of NaCl in the recovery medium on the survivors of *E. coli*. The cells were heated at 55 °C in M/10 phosphate buffer at pH 7.0. 1: plate-counted on a nutrient agar. 2: plate-counted on a nutrient agar plus 5% NaCl

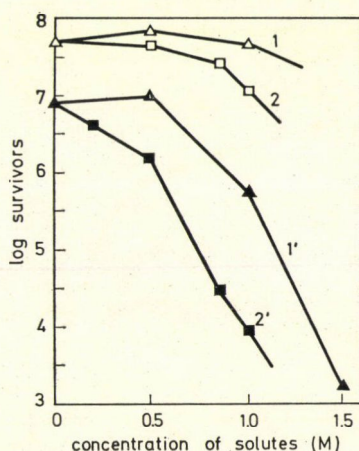


Fig. 5. Effect of KCl and sucrose in the recovery medium on the survivors of *E. coli*. The cells were heated at 55 °C for 3 min in M/10 phosphate buffer at pH 7.0. 1, 1': unheated or heated, respectively, plate-counted on a nutrient agar plus sucrose. 2, 2': unheated or heated, respectively, plate-counted on a nutrient agar plus KCl



of inhibition of cell growth, it has been found that many chemicals among the food preservatives, disinfectants, antibiotics and other additives had significant effects on the thermal destruction of microorganisms. Thus the authors have conducted detailed studies of the environmental factors affecting, and the mechanism of the enhancing effect of sodium chloride, tylosin and sorbic acid on the thermal injury of *E. coli* and *C. utilis* inferring the two separated mechanisms: 1) the acceleration of the thermal injury during heating, 2) the inhibition of recovery process by chemicals.

## 2.2. Combined effect of sodium chloride

Sodium chloride is a common component of foods and is added to various foods for the purpose of processing. The effect of sodium chloride on the cells of microorganisms during heating is not regular. It has been shown by several workers (BEUCHAT & LECHOWICH, 1968; BRIGGS & YAZDANY, 1970) that sodium chloride had either a protective or an accelerating effect on the thermal destruction of microorganisms. In this study, sodium chloride was added to the heating menstruum and the heated cells were plate-counted using a proper recovery medium in the absence or presence of sodium chloride at 5 or 7%. Sodium chloride in the heating menstruum was found to protect against thermal stress in the cells of *C. utilis* and *E. coli* depending on a concentration of sodium chloride as shown in Table 2. But it was already shown in Figs. 2, 3 and 4 that sodium chloride inhibited significantly the recovery process of the thermal injury of *C. utilis* and *E. coli*.

Fig. 6 indicates the reduction of the viable cell counts of *E. coli* in the presence of sodium chloride during heating and recovery treatment.

On the basis of these data, it was indicated that the heat-treated cells lost the sodium chloride tolerance and therefore could not form colonies on the recovery medium containing a relatively high concentration of sodium

Table 2

*Effect of NaCl in the heating menstruum on the thermal destruction of C. utilis*

Concentration of NaCl (%) in M/10 phosphate buffer at pH 4.0	D value in minutes
0	7.1
2	7.4
4	6.3
7	9.7
10	31.1

The cells were heated at 50 °C for 10 min; D value indicates 90% destruction time.

chloride. When, however, sodium chloride is present both in the heating menstruum and in the recovery medium, the reduction of viable cell counts was hardly observed, as shown in Fig. 6. It is obvious from these results that the protective effect of sodium chloride is significant against intracellular substances during heating.

The factors affecting the inhibiting effect of sodium chloride upon the recovery process were examined. The thermally injured cells of *C. utilis* and *E. coli* were repaired by incubation in the culture media (rich or minimal medium) or phosphate buffer at optimal temperature under shaking conditions.

Some results of the recovery of salt tolerance of heat-injured cells were shown in Figs. 7, 8 and 9. Fig. 7 indicates the time course of recovery of sodium chloride tolerance of the heat-injured cells of *C. utilis* and Fig. 8 indicates the recovery of NaCl, KCl and sucrose tolerance of heat-injured cells of *E. coli*. Fig. 9 indicates the effect of the composition of the incubating medium on the recovery of heat-injured cells of *E. coli*. The heat-injured cells of *C.*

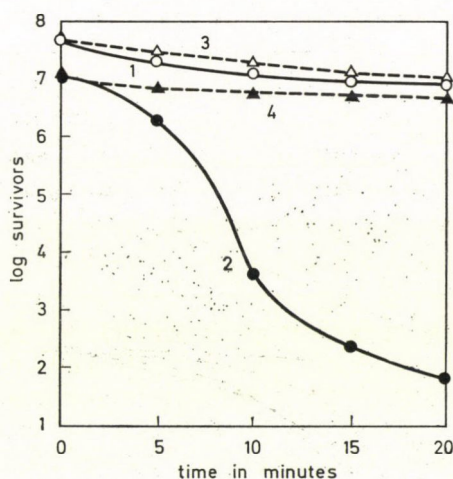


Fig. 6. Effect of NaCl in the heating menstruum and the recovery medium on the thermal destruction of *E. coli*. The cells were heated at 50 °C in the heating menstruum containing 0.8 or 5.0% NaCl, and plate-counted on a nutrient agar (NaCl: 0.5%) or one plus 5.0% NaCl

No.	Concentration of NaCl (%)	
	in the heating menstruum	in the recovery medium
1	0.8	0.5
2	0.8	5.0
3	5.0	0.5
4	5.0	5.0



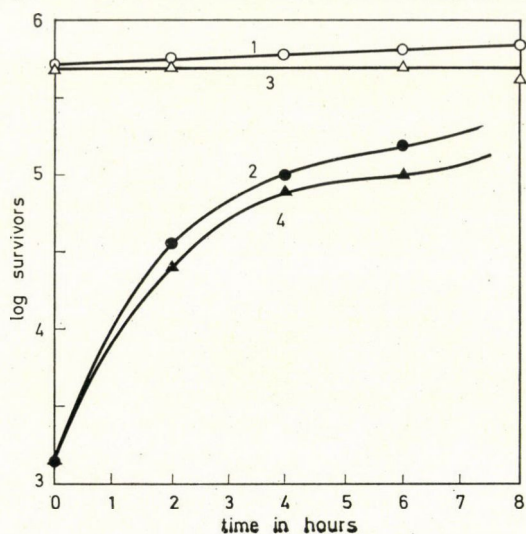


Fig. 7. Recovery of heat-injured cells incubated in CDPY medium or M/10 phosphate buffer. The cells were heated at 45 °C for 15 min.

No.	Medium	
	for incubation	for plate-counts
1	CDPY	CDPY agar
2	CDPY	CDPY plus 7% NaCl agar
3	Phosphate buffer	CDPY agar
4	Phosphate buffer	CDPY plus 7% NaCl agar

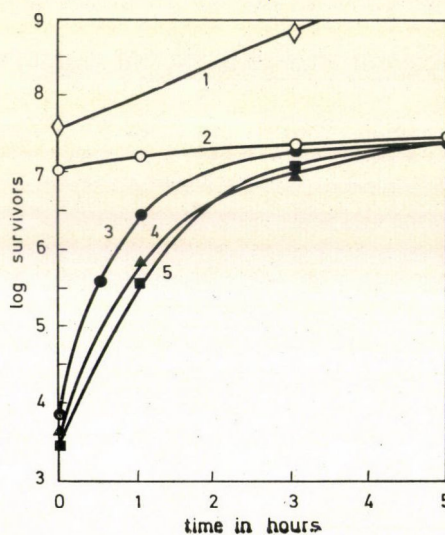


Fig. 8. Recovery of solute tolerance of heat-injured cells of *E. coli*. 1: unheated; 2, 3, 4, 5: heated at 55 °C for 3 min, incubated at 37 °C in the minimal medium, and plate-counted on a nutrient agar containing no solute, 5% NaCl, 0.855 M KCl, or 1.32 M sucrose, respectively. The minimal medium consists of 2.5 g Na-citrate, 2.0 g glycerol, 5.0 g  $(\text{NH}_4)_2\text{SO}_4$ , 1.0 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g  $\text{KH}_2\text{PO}_4$  and 1.0 g NaCl per 1000 ml

*utilis* and *E. coli* were repaired in the culture medium and also repaired in a salt solution or phosphate buffer in the same way, but this repair was inhibited in glycerol and sodium citrate solution.

Furthermore, experiments were conducted to test the effect of the addition of some inhibitors upon the repair of salt tolerance of *E. coli*. The

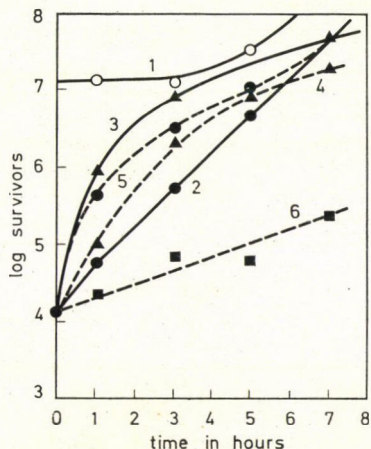


Fig. 9. Effect of medium composition on the recovery of heat-injured cells of *E. coli*. The cells were heated at 55 °C for 3 min, incubated at 37 °C in the following media under shaking conditions and plate-counted on a nutrient agar plus 5% NaCl. 1: control; 2: nutrient broth; 3: minimal medium; 4, 5, 6: minimal medium, omitted salts, carbon and nitrogen sources, or salts and nitrogen source, respectively

results obtained were shown in Fig. 10 a,b, c & d. All inhibitors employed inhibited the repair of salt tolerance, but the inhibition of KCN, 2,4-DNP and furylfuramide was particularly significant.

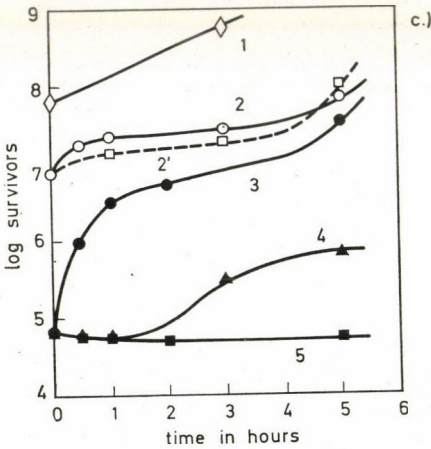
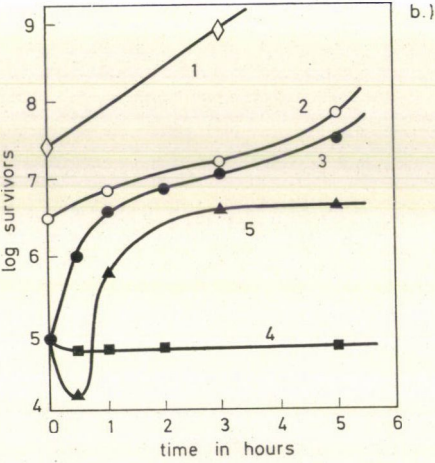
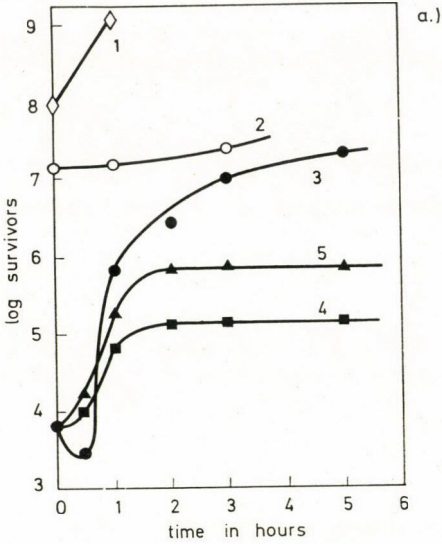
Thus it can be suggested that protein synthesis and ATP synthesis are required for the recovery process of thermally injured cells of *E. coli*, while in the case of *C. utilis* the biosynthesis of RNA and protein is essential (TSUCHIDO *et al.*, 1972a).

### 2.3. Combined effects of tylosin on the thermal destruction of microorganisms

As the damage of the cell membrane is induced by sublethal heat in general, there is a possibility that tylosin may permeate the membrane of heated cells and inhibit cell growth.

It was previously indicated by LATEGAN and VAUGHN (1964) that when *Sal. typhimurium* in whole egg at pH 5.5 and 55 °C was heated in the presence of tylosin at 30 and 1 000 µg/ml, 13 and 27.6% reductions in D value were observed, respectively. When *E. coli* was heated at 50 °C in M/10 phosphate buffer (pH 7.0), 20 to 40% reduction in D value was observed.





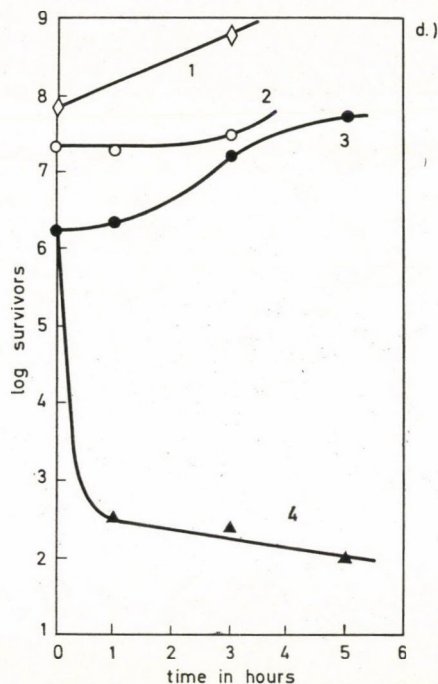


Fig. 10. Effect of inhibitors on the recovery of heat-injured cells of *E. coli*. The cells were heated at 55 °C for 3 min, incubated at 37 °C in the minimal medium, and plate-counted on a nutrient agar with (3, 4, 5) or without (2, 2') 5% NaCl. The following inhibitors were pre-added to the minimal medium. 1: unheated. a) 3: none; 4: tylosin, 200 µg/ml; 5: chloramphenicol, 100 µg/ml. b) 3: none; 4: 2,4-dinitrophenol, 75 µg/ml; 5: 5-fluorouracil, 100 µg/ml. c) 3: none; 4: tylosin, 400 µg/ml; 5: 0.001 M KCN; 2' 0.01 M KCN. d) 3: none; 4: furylfuramide, 1 µg/ml

Figs. 11 and 12 indicate the results that the growth of heat treated cells of *Ps. aeruginosa* at 53 °C and *E. coli* at 55 °C is inhibited by the addition of tylosin at 20 µg/ml. As the time of addition of tylosin was delayed, the degree of growth inhibition by tylosin markedly decreased as shown in Fig. 12. The effect of tylosin on the survivors of *E. coli* heated at 55 °C for 3 min was observed and it was found that 90% or more cells lost the colony forming ability in the presence of tylosin ranging from 200 to 800 µg/ml as shown in Fig. 13. Fig. 14 indicates the results that tylosin tolerance of the heat-injured cells of *E. coli* are repaired in a short period by incubation under suitable conditions.

These results indicate the evidence that the thermal damage in the heat-treated cells of *E. coli* occurs in the cell membrane. Therefore tylosin can be used as a useful means in the study on the thermal injury of bacterial cells and it is also useful as a model system in the study of a combined effect of mild heating and chemicals. From these results it is expected that tylosin



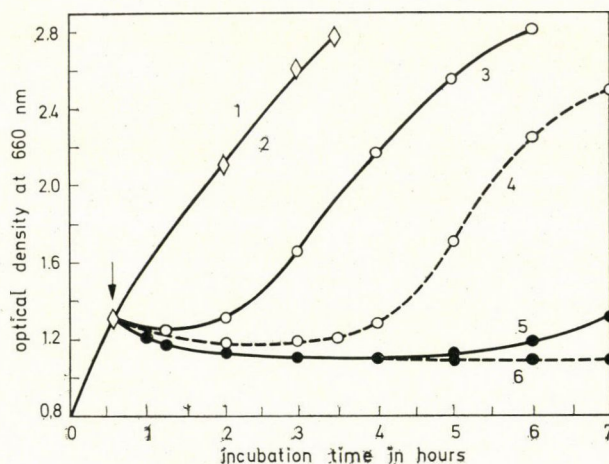


Fig. 11. Effect of tylosin on the growth of heat-treated cells of *Ps. aeruginosa*. The cells were heated at 53 °C in a nutrient broth, incubated at 30 °C under shaking conditions, sampled periodically, measured for optical density at 660 nm. Tylosin was added after heat treatment at a concentration of 20 µg/ml. 1: control; 2: unheated, added 20 µg/ml tylosin; 3: heated for 3 min; 4: heated for 5 min; 5: heated for 3 min, added 20 µg/ml tylosin; 6: heated for 5 min, added 20 µg/ml tylosin

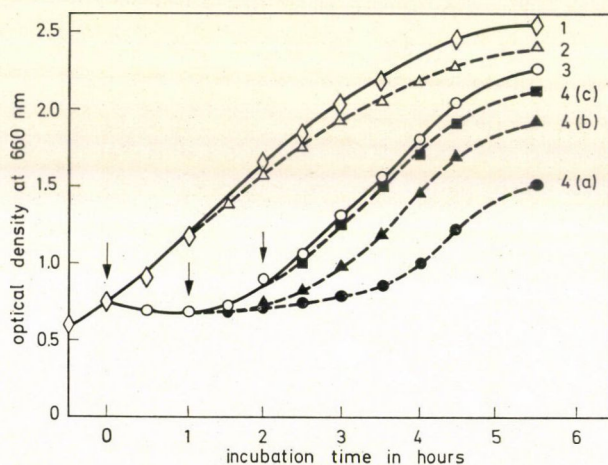


Fig. 12. Effect of tylosin on the growth of heat-treated cells of *E. coli*. Tylosin was added at 0 hr (a), 1 hr (b) and 2 hrs (c) after heat treatment at 55 °C for 3 min. The arrows indicate the time of addition of 20 µg/ml tylosin. 1: control; 2: unheated, added tylosin; 3: heated; 4: heated, added tylosin

can inhibit the growth of Gram-negative bacteria survived in mildly heated food. The detailed study is in progress.

#### 2.4. Combined effect of sorbic acid on the thermal destruction of *C. utilis*

Sorbic acid does not act synergistically against the thermal destruction of *Bacillus* spores, but acts against other microorganisms. These results were summarized in Table 3.

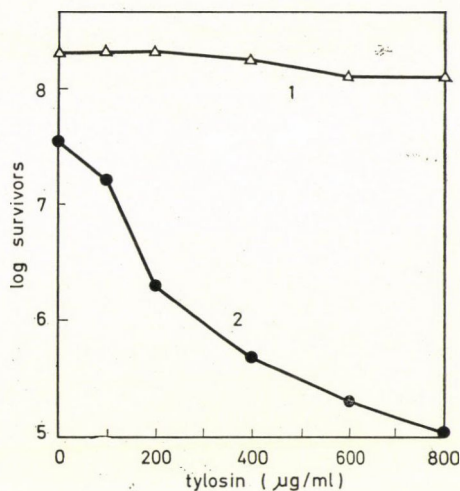


Fig. 13. Effect of tylosin on the survivors of *E. coli* heated at 55 °C for 3 min in M/10 phosphate buffer at pH 7.0. 1: unheated; 2: heated at 55 °C for 3 min. The cells were plate-counted on a nutrient agar with or without tylosin at various concentrations

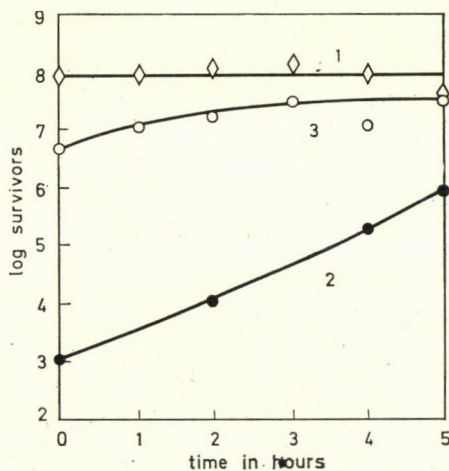


Fig. 14. Recovery of tylosin tolerance of heat-injured cells of *E. coli*. 1: unheated; 2, 3: heated at 55 °C for 3 min, plate-counted on a nutrient agar with or without tylosin 200 μg/ml, respectively



Table 3

Combined effect of sorbic acid on the thermal destruction of microorganisms under various conditions

Test organism	Heating condition	% reduction in D value
<i>Bacillus subtilis</i> spores	90 °C, pH 7.0	0
<i>E. coli</i>	55 °C, pH 5.5	61
<i>Sal. typhimurium</i>	55 °C, pH 5.5	60
<i>C. utilis</i>	50 °C, pH 4.0	81
<i>Han. anomala</i>	50 °C, pH 4.0	83.4
<i>S. rouxii</i>	50 °C, pH 4.0	73.6
<i>S. cerevisiae</i>	50 °C, pH 4.0	79.2
<i>Asp. niger</i> conidia	50 °C, 60 °C, pH 4.0	21.42
<i>Pen. thomii</i> conidia	50 °C, 60 °C, pH 4.0	0

As it was found that sorbic acid had a significant combined effect with heating particularly against yeast, the system of sorbic acid and *C. utilis* was used as a model system for evaluating the mechanism of the enhancing effect of chemicals on the thermal destruction of microorganisms.

The factors affecting the combined effect of sorbic acid were determined at first. The combined effect of sorbic acid was scarcely observable when incubating at 30 °C for 300 min, but it was markedly observed at temperatures above 45 °C. A linear relationship was obtained between the D value and heating temperature ranging from 45° to 60 °C (Fig. 15). The enhancing effect of sorbic acid was dependent upon the concentration of sorbic acid at a concentration below 0.1% but did not increase at 0.2%.

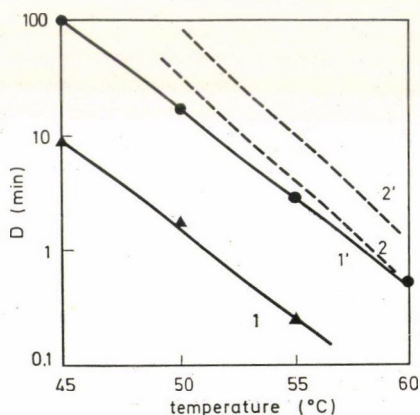


Fig. 15. Relationship between D value and heating temperature. 1, 1': *C. utilis* cells heated in phosphate buffer (pH 4.0) with or without 0.085% sorbic acid. 2, 2': *Sal. typhimurium* cells heated in liquid whole egg (pH 5.5) with or without 0.1% sorbic acid

The undissociated form was considered to be the antimicrobially active form of sorbic acid and so it has been observed that the antimicrobial activity of sorbic acid was dependent upon the pH of the medium. Although it was found that the heat resistance of *C. utilis* was independent of the pH of the medium, the enhancing effect of sorbic acid was more significant as the pH decreased. A linear relationship was obtained between the lethal rate and the pH of the medium. Accordingly, it may be concluded that the enhancing effect of sorbic acid on the thermal destruction of *C. utilis* is due to the undissociated molecule. The enhancing effect of sorbic acid was not affected significantly by other factors such as cell age, composition of the suspending menstruum and the recovery medium.

The following experiments were conducted to study the mechanism of the enhancing effect of sorbic acid on the thermal destruction of *C. utilis*. The time course of growth of heated cells at 45 °C for 10 min with sorbic acid in a low concentration of 0.005% was observed. These results obtained showed essentially the same tendency as Figs. 11 and 12. It was also found in the heat-treated cells of *C. utilis* that the thermal injury occurs on mild heating with sorbic acid and is repaired within a short time by incubation under suitable conditions.

Table 4 indicates the effect of sorbic acid on the growth of heated cells in a suspending menstruum containing sorbic acid at 0.005%. The inhibition of growth was compared by the prolongation of lag time. The lag time in the presence of sorbic acid at 0.005% during heating was 19 hours and, on the

Table 4  
*Effect of sorbic acid on the growth of heat-treated cells of C. utilis*

Treatment	Addition of 0.005% sorbic acid during the recovery	Lag time (hr)
Control	—	7
	+	6
Added 0.005% sorbic acid, unheated	—	11
	+	7
Heated without sorbic acid	—	11
	+	28
Heated with 0.005% sorbic acid	—	19
	+	34

The cells heated at 50 °C for 5 min in M/10 phosphate buffer (pH 4.0) with or without 0.005% sorbic acid were centrifuged and washed to remove added sorbic acid. Thereafter these cells were suspended in Czapek—Dox's medium with or without 0.005% sorbic acid, incubated at 30 °C under shaking conditions, sampled periodically, measured for optical density at 600 nm, and the lag time in hours calculated.



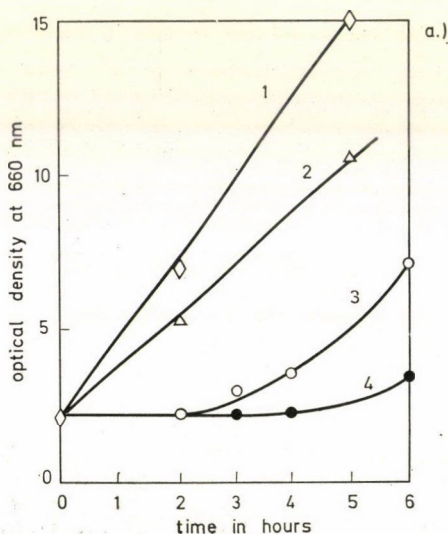
contrary, 28 hours in the presence of sorbic acid after heating. Accordingly it may be suggested that sorbic acid acts more effectively after heating than during heating.

It was found that sorbic acid did not accelerate the leakage of intracellular substances such as orcinol reacting materials and pre-incorporated  $^{14}\text{C}$ -amino acids and that the leakage and degradation of RNA in the heated cells were not influenced by sorbic acid (TSUCHIDO *et al.*, 1972b).

The recovery rate of salt tolerance of heat-treated cells was not different in the presence or absence of sorbic acid at a 0.005% level (TSUCHIDO *et al.*, 1972b).

Furthermore, it was indicated that the uptake of  $^3\text{H}$ -sorbic acid into the cells was not accelerated by mild heating, although it was increased with decreasing the pH of the medium.

Fig. 16 indicates the time course of macromolecular synthesis in the recovery and growth of heat-treated cells of *C. utilis*. At first the incorporation of  $^{14}\text{C}$ -adenine into the RNA fraction of heat-treated cells was initiated, the incorporation of  $^{14}\text{C}$ -adenine into the DNA fraction followed it and finally the biosynthesis of protein occurred and this corresponded to the increase in optical density at 660 nm. When comparing with the repairing behaviour of cells heated with or without sorbic acid, the incorporation of  $^{14}\text{C}$ -adenine into the RNA or DNA fraction was delayed in the cells heated with sorbic acid and furthermore the protein synthesis in the heated cells with sorbic acid was completely inhibited for 5 hours or more. It was similar when 5  $\mu\text{g}/\text{ml}$  of cycloheximide (minimal inhibitory concentration against *C. utilis*) was added to the recovery medium.



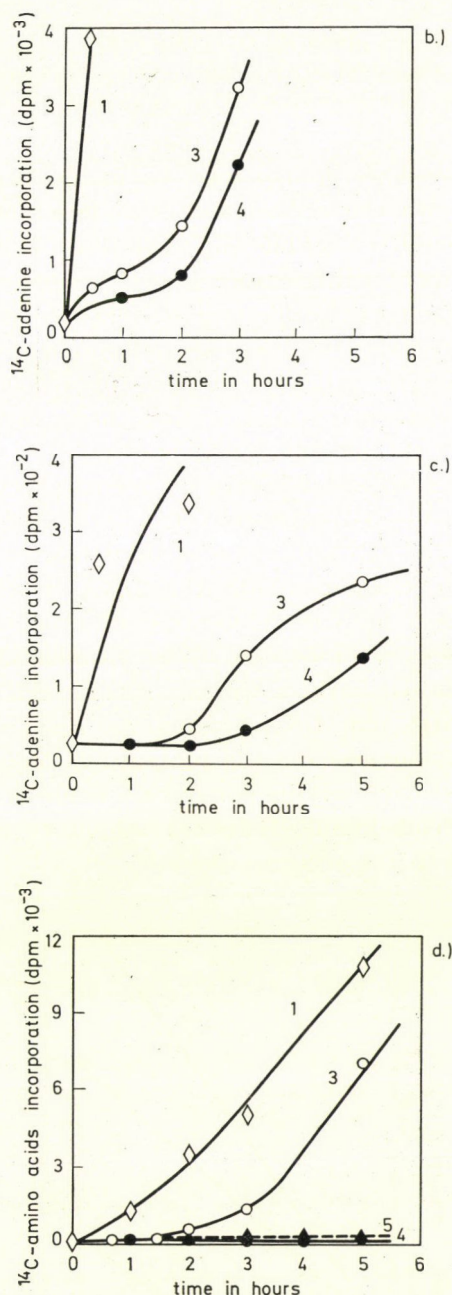


Fig. 16. Intracellular macromolecule synthesis during the recovery of heat-injured cells of *C. utilis* with or without sorbic acid. The cells were heated at 45 °C for 15 min with or without 0.005% sorbic acid and incubated at 30 °C. a) Growth, b) RNA synthesis, c) DNA synthesis, d) Protein synthesis. 1: unheated; 2: unheated, added sorbic acid; 3: heated without sorbic acid; 4: heated with sorbic acid; 5: heated without sorbic acid, incubated with 5  $\mu$ g/ml cycloheximide



The time course of the respiratory activity is indicated in Fig. 17.  $Q_{O_2}$  values of unheated cells with or without sorbic acid were 273 and 229  $\mu\text{l/hr/mg}$ , respectively. The respiratory activity in heated cells without sorbic acid was repaired and increased after a 1-hour induction period, but the activity in heated cells with sorbic acid was hardly repaired even after a 5- to 6-hour period.

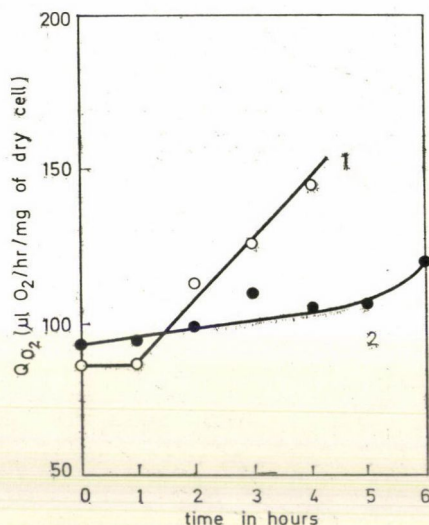


Fig. 17. Effect of sorbic acid on the oxygen uptake of heat-injured cells of *C. utilis*. 1: heated at 45 °C for 15 min in CDPY medium. 2: heated at 45 °C for 15 min in CDPY medium with 0.005% sorbic acid

It is shown from the results obtained that marked reductions of the protein synthesis and respiratory activity occur in the heated cells of *C. utilis* with 0.005% of sorbic acid.

### 3. Conclusions

Although the intracellular substances were released from the heat-treated cells of *C. utilis* and *E. coli*, the amount of leakage was not always proportional to the loss in viability of cells in this study. Therefore, these results did not indicate the quantitative evidence of the cell membrane damage caused by exposure to high temperature. It seems reasonable to infer that leakage is a secondary effect, occurring as a result of cellular degradation or membrane damage.

The increased sensitivity to NaCl, KCl and sucrose at relatively high concentrations was proved in the heated cells of *E. coli* and *C. utilis*, similar

to *Staph. aureus* (IANDOLO & ORDAL, 1966), *Strept. faecalis* (CLARK *et al.*, 1968) and *Sal. typhimurium* (CLARK & ORDAL, 1969).

But, as it was found in this experiment, that the heat-injured cells were similarly repaired within a short period by incubation in the culture media (rich or minimal) and in a salt solution or phosphate buffer solution, the mechanism of the repair process of *E. coli* and *C. utilis* seems to be different from those of *Staph. aureus* (IANDOLO & ORDAL, 1966), *Strept. faecalis* (CLARK *et al.*, 1968), and *Sal. typhimurium* (TOMLINS & ORDAL, 1971) and it seems to be rather similar to the results obtained with the heated conidia of *P. expansum* (BALDY *et al.*, 1970).

The addition of the metabolic inhibitors, such as chloramphenicol, tylosin, 5-fluorouracil, 2,4-dinitrophenol, KCN to the incubating medium showed that the recovery process of *E. coli* was dependent on adenosine triphosphate synthesis and protein synthesis.

These results are similar to those obtained with *Sal. typhimurium* (TOMLINS & ORDAL, 1971), while it was established that RNA synthesis and the synthesis of new protein seemed to be essential for the repair of the heated cells of *C. utilis* (TSUCHIDO *et al.*, 1972a).

The addition of NaCl after heating indicated a significant enhancing effect and, on the contrary, during heating showed a markedly protective effect on the thermal destruction of *E. coli* and *C. utilis*. But when the cells were heated and counted in the presence of a relatively high concentration of NaCl, the protective effect predominated over the enhancing effect after heating as shown in Fig. 6.

The inefficiency of tylosin against Gram-negative bacteria was explained as due to its membrane permeability (SUZUKI *et al.*, 1970; KAGEYAMA *et al.*, 1971). On the basis of this study it seemed reasonable to assume that tylosin may permeate the cell membrane during the heating or the recovery process and inhibit the protein synthesis by the binding with the ribosome.

The enhancing effect of sorbic acid on the thermal destruction was proved in a relatively wide range of microorganisms. Thus the system of sorbic acid and *C. utilis* was used as a model system for evaluating the mechanism of the enhancing effect of chemicals on the thermal destruction of microorganisms.

It was found that sorbic acid has a greater effect on the inhibition of the cellular repair mechanism after heating and its effect is due to the inhibition of the synthesis of new protein and the respiratory activity during the repair process. Although the mode of action of sorbic acid has not been investigated in detail, the inhibition of the dehydrogenase system and oxidative phosphorylation similar to 2,4-dinitrophenol was described by MELNICK *et al.* (1954); WHITAKER (1959), and YORK and VAUGHN (1964). The authors



have found that the protein synthesis of *C. utilis* is markedly inhibited by the minimal inhibitory concentration of sorbic acid (0.005%) (not presented).

From these results it is suggested that the cells of *C. utilis* become more sensitive to sorbic acid on mild heating and sorbic acid acts markedly during the recovery process and the lag time of the growth of cells is prolonged or the viable cell counts are decreased by the loss of the colony-forming activity.

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## EFFECT OF WATER ACTIVITY AND pH ON STAPHYLOCOCCAL ENTEROTOXIN *B* PRODUCTION\*.

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The effects of pH level and variation in water activity ( $a_w$ ) of test media on enterotoxin *B* production and growth of *Staphylococcus aureus* S-6 were investigated. Adjustment of pH to 5.6 and 6.8 did not influence growth or toxin production under the conditions investigated. Water activity reduction, obtained by the addition of two different solute systems, reduced total and relative enterotoxin *B* production. Growth as determined by extension of lag phase and maximal numbers was also inhibited by  $a_w$  reduction, however, the effect of  $a_w$  limitation on toxin production was more pronounced than on growth.

The staphylococci and particularly *Staphylococcus aureus* constitute a group of bacteria that are highly tolerant of limited moisture conditions. Thus, staphylococci have been of special significance in salt-cured meats and curing brines where they are frequently implicated in outbreaks of food-borne disease. Recent emphasis on the development of intermediate moisture foods has further shown the need for a greater understanding of moisture limitation on the growth activities of this organism. SCOTT (1953) was the first researcher to apply the broadly applicable water activity or  $a_w$  term to quantitate the effect of water condition in a medium on staphylococcal growth when he noted that growth occurred over an  $a_w$  range of 0.86 to 0.999. Growth was further found to be relatively independent of the solute used to adjust water activity. Additional reports appearing in the literature have essentially confirmed Scott's findings, however, a recent article (LABUZA *et al.*, 1972) showed growth of *S. aureus* at  $a_w$  0.83, somewhat below the previously described minimum of 0.86.

Most of the studies emphasizing the influence of water limitation on enterotoxin production have been carried out utilizing NaCl to limit water activity, expressing the quantity of solute added on a weight per cent basis. Examples of these studies are reports by GENIGEORGIS and SADLER (1966), McLEAN *et al.* (1968), and HOJVAT and JACKSON (1969). These reports conclude that enterotoxin production is restricted by increasing NaCl concentrations

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and inhibition of toxin formation appears to be more sensitive to increase in solute levels than growth of viable cells. Although agreeing that total toxin levels were reduced as water was limited, MARKUS and SILVERMAN (1970) determined that the relative amount of enterotoxin *A* produced per unit of cell growth was not inhibited by increasing NaCl concentrations, thus raising the possibility that the toxin depletions noted above were the result of inhibition of cell growth rather than an inherent effect on toxin synthesis.

Studies in our laboratories (TROLLER, 1971) have utilized the  $a_w$  term to describe the effect of water limitation of adjusted media on staphylococcal growth and enterotoxin *B* production. Growth rates and maximal cell yields were reduced as the  $a_w$  was lowered, however, the most profound effect was produced on enterotoxin levels in the medium. Very slight water activity suppression was sufficient to cause an inordinately large reduction in toxin formation in media adjusted with two different solute systems. Subsequent studies with an enterotoxin *A*-producing strain (TROLLER, 1972) noted a similar, but less dramatic decrease in toxin levels.

The present studies have as their objective the determination of the presence of interactions between pH and  $a_w$  levels and the effect of this interaction on staphylococcal growth and toxin formation.

## 1. Materials and methods

### 1.1. Cultures and growth conditions

The methods used in these studies are primarily those described in our previous reports (TROLLER, 1971, 1972). The S-6 strain, standard toxins, and antisera employed throughout this work were gifts of *M. S. Bergdoll*, Food Research Institute, University of Wisconsin. The medium used was described by RIESER and WEISS (1969) and contained 3% each of a partially hydrolyzed protein (PHP, Mead Johnson and Company, Evansville, Indiana) and NZ Amine NAK (Sheffield Chemical Company, Norwich, N. Y.). Filter sterilized thiamine and nicotinic acid in small amounts were also added following autoclaving.

The relative composition of media with regard to solute concentration and respective  $a_w$  levels are shown in Table 1. The salt mixture consists of a 5 : 3 : 2 ratio mixture of NaCl : KCl :  $\text{Na}_2\text{SO}_4$ . Each of the media shown in this table were adjusted to pH 5.6 or 6.8. Growth was followed by withdrawing aliquots at various times for counting on Plate Count Agar (Difco).



Table 1

*Relationship between solute content of basal medium and water activity ( $a_w$ )*

Solute	$a_w$ adjustment % solute added	$a_w$
NaCl	0 (Control)	0.99
	3	0.97
	4	0.94
	7.5	0.92
	10	0.90
Mixed salt (NaCl : KCl : Na <sub>2</sub> SO <sub>4</sub> ) (5 : 3 : 2)	0 (Control)	0.99
	3	0.96
	6	0.94
	8	0.92
	10	0.90

### 1.2. Enterotoxin analysis

Aliquots containing 20 ml each of the culture broth were withdrawn from the growth flasks at the indicated times, followed by extraction and concentration. These procedures were carried out by refrigerated centrifugation at 45 000  $\times g$  for 15 minutes to remove cells, followed by dialysis for 24 hours against distilled water at 5 °C. The dialysis bags were then transferred to a 50% water solution of polyethylene glycol 20 M (Union Carbide Corporation, Chicago, Illinois) and the contents of the sack were concentrated at 5 °C overnight. The concentrated material was removed from the dialysis bags by rinsing with 2.0 ml of 0.37% Brain Heart Infusion broth (Difco) containing a small amount of merthiolate and centrifuged again under the conditions stated previously.

The supernatant was analyzed for enterotoxin B by the single gel diffusion tube method as described by OUDIN (1952) and modified by WEIRETHER *et al.* (1966). The minimal amount of toxin detectable by this procedure was 1  $\mu\text{g/ml}$ .

### 1.3. Humidity measurements

Water activity estimations are expressed as  $a_w$  or the ratio of the vapour pressure of a given solution to that of pure water.  $a_w$  can also be expressed as the equilibrium relative humidity, divided by 100, of an atmosphere above a given material in a closed chamber. Further information on the basis and derivation of the  $a_w$  term is contained in the review by SCOTT (1957).

Water activity levels of the various media were obtained by placing approximately 20 to 30 ml of the test solution in a 4 oz. jar, sealed with a cap through which protruded a sensing element connected to a direct reading galvanometer. The element is a LiCl<sub>2</sub> type unit manufactured by Hygrodynamic-



ics, Inc., Silver Springs, Maryland. All elements were calibrated against saturated solutions of known  $a_w$  levels immediately before measurements were taken. Samples were allowed to equilibrate for 2 hours at 30 °C before testing.

#### 1.4. Known enterotoxin controls

Enterotoxin B in known amounts was mixed with uninoculated control media adjusted to various  $a_w$  levels. The concentration and analysis procedures were identical to those described for inoculated media except that the initial centrifugation and decantation steps were omitted. The enterotoxin-containing controls were maintained at 30 °C for 24 hours before analysis for the toxin. Recovery of enterotoxin from these media ranged from 93 to 100%, suggesting that there was no direct action of low  $a_w$  on the enterotoxin itself and that the recoveries obtained were similar to those obtained from experimental media.

## 2. Results

A growth curve of *S. aureus* S-6 in the medium adjusted to an initial pH level of 5.6 and poised at various  $a_w$  levels by means of the salt mixture previously described is shown in Fig. 1. This figure shows that growth rate, lag phase, and to a lesser degree, maximal numbers are affected by lowered  $a_w$  levels.

Following 48 hours of incubation, all counts exceeded  $1 \times 10^9$ /ml, a level of growth that could be expected to support extensive enterotoxin production in this medium. Because there was little variation between growth curves with respect to solute or pH effects, these data in the remaining three media are not included.

The relationship of lag phase duration to water activity and pH of the medium is shown in Table 2. A marked extension of lag times occurred with reduction in  $a_w$ , however, pH level and solute type appeared to have little influence on the duration of the growth phase. The maximal observed lag phase times, 9 to 18 hours, occurred at the lowest  $a_w$  tested.

During incubation, a strong, upward shift in pH was noted as growth proceeded. This shift occurred primarily during logarithmic growth and extended into the maximum stationary phase. The highest pH levels attained, 8.5 and 8.8, occurred in the control or high  $a_w$  media, however, neither initial pH levels nor solute type influenced the final pH of the medium. This upward shift in pH is probably the result of deamination of the abundant amount of peptides present in this medium and could not be eliminated by the addition of buffers.

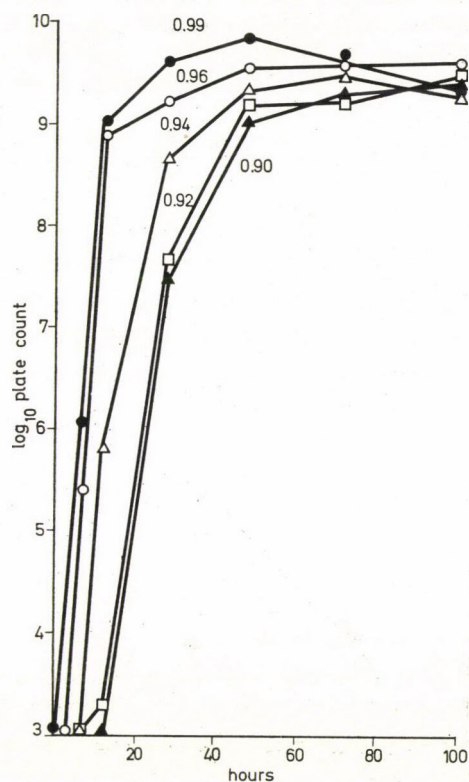


Fig. 1. Effect of  $a_w$  on growth of *S. aureus* S-6.  $a_w$  of medium adjusted with salt mixture. Initial pH 5.6, incubation at 37 °C

Table 2

Effect of pH and  $a_w$  of media on lag phase

$a_w$	NaCl*		Salt mixture* (NaCl : KCl : Na <sub>2</sub> SO <sub>4</sub> )	
	Lag time, hours			
	pH 5.6**	pH 6.8	pH 5.6	pH 6.8
0.99	1	1	2	1.5
0.97	2.5	2	—	—
0.96	—	—	5	2.5
0.94	6	2.5	7	4
0.92	7.5	5	8	4
0.90	12	13	18	9

\* Solute employed to adjust medium.

\*\* Represents initial pH of medium.



The effect of  $a_w$  on enterotoxin *B* production in the four test media is shown in Figs. 2, 3, 4, and 5. Toxin yields in media in which NaCl was utilized to adjust  $a_w$  as shown in Figs. 2 (pH 5.6) and 3 (pH 6.8), were significantly lower than when the mixed solute, as shown in Figs. 4 (pH 5.6) and 5 (pH 6.8) was present, thus indicating the presence of a strong solute effect. The level of toxin present in each case directly correlated with the  $a_w$  of the medium. Maximal enterotoxin *B* yields were observed at maximal  $a_w$  levels in all media. As observed above in the data relating growth to  $a_w$ , no influence of initial pH could be detected.

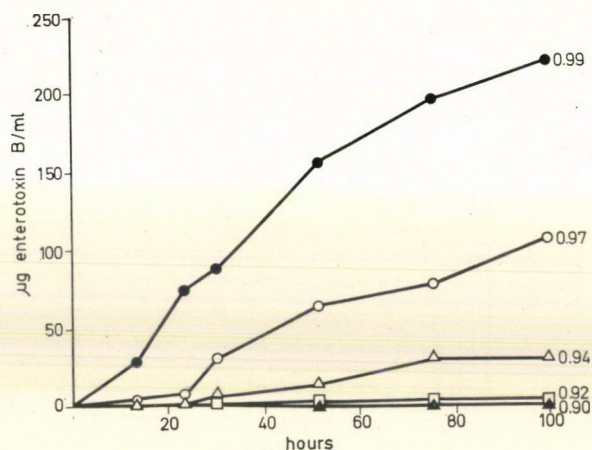


Fig. 2. Effect of  $a_w$  on enterotoxin *B* production in medium adjusted with NaCl, pH 5.6

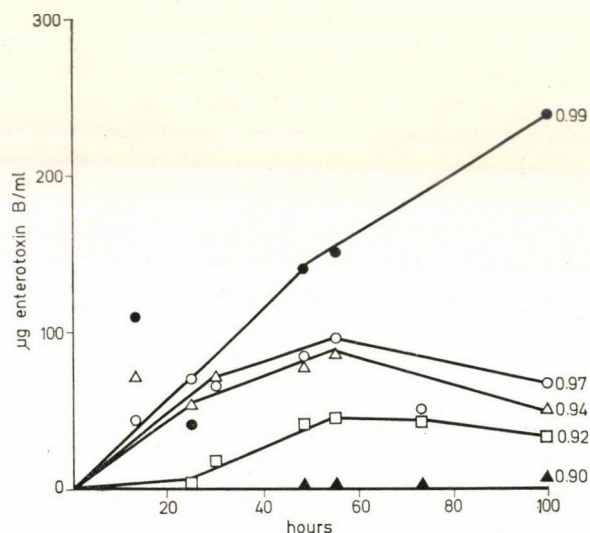


Fig. 3. Effect of  $a_w$  on enterotoxin *B* production in medium adjusted with NaCl, pH 6.8

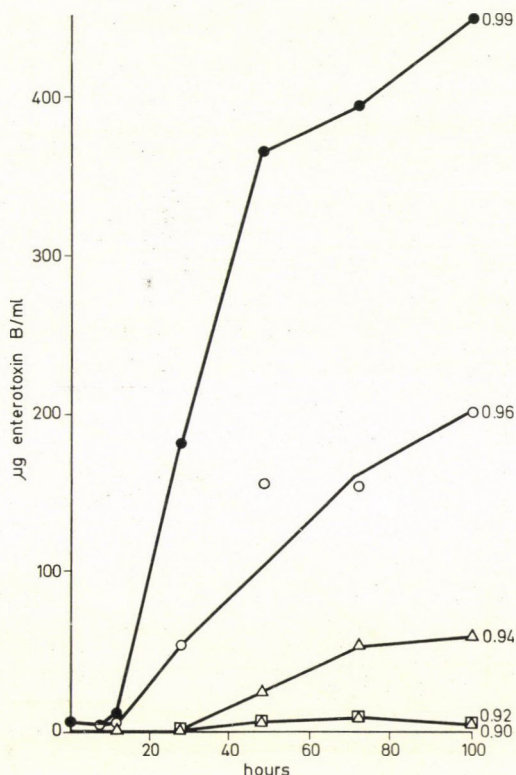


Fig. 4. Effect of  $a_w$  on enterotoxin *B* production in medium adjusted with mixed solutes, pH 5.6

The effect of  $a_w$  on maximal toxin yields is shown in Fig. 6. The highest yields were obtained at  $a_w$  0.99, however, decreases in  $a_w$  of the medium brought about lower levels of enterotoxin *B*. The minimal  $a_w$  tested, 0.90 which corresponded to a solute concentration of 10%, resulted in the presence of very low levels of enterotoxin despite the presence of  $>10^9$  staphylococci per ml.

Although the effect of  $a_w$  on lag phase, growth rate and enterotoxin production was pronounced, the effect on maximal plate counts was not great. To determine if the suppressed enterotoxin levels were due merely to fewer viable cells in a given medium, maximal enterotoxin levels obtained per  $10^8$  cells were related to water activity. The data in Fig. 7 show that the inhibiting effect of  $a_w$  on enterotoxin *B* production exists independently of total plate count in the medium. Thus, reduction in  $a_w$  adversely affects the amount of enterotoxin produced per cell. As expected, the solute differences between the mixed salt solute system and NaCl are obvious and as noted above, there is little difference in response to variation in initial pH.



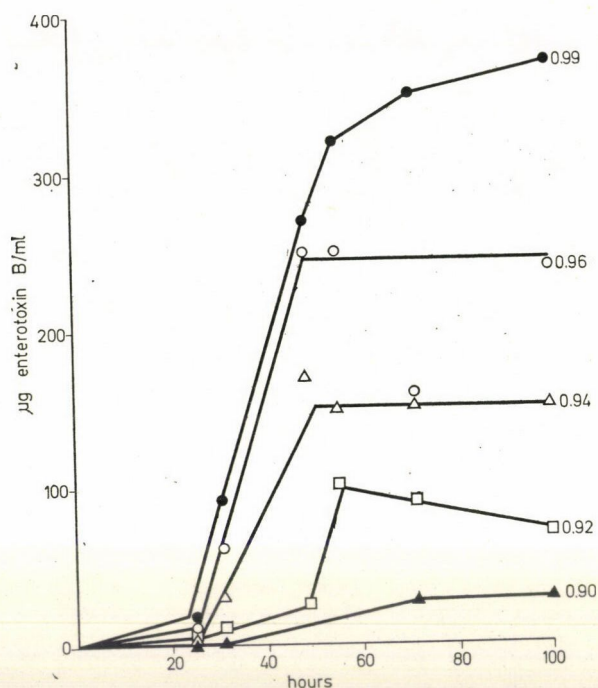


Fig. 5. Effect of  $a_w$  on enterotoxin *B* production in medium adjusted with mixed solutes, pH 6.8

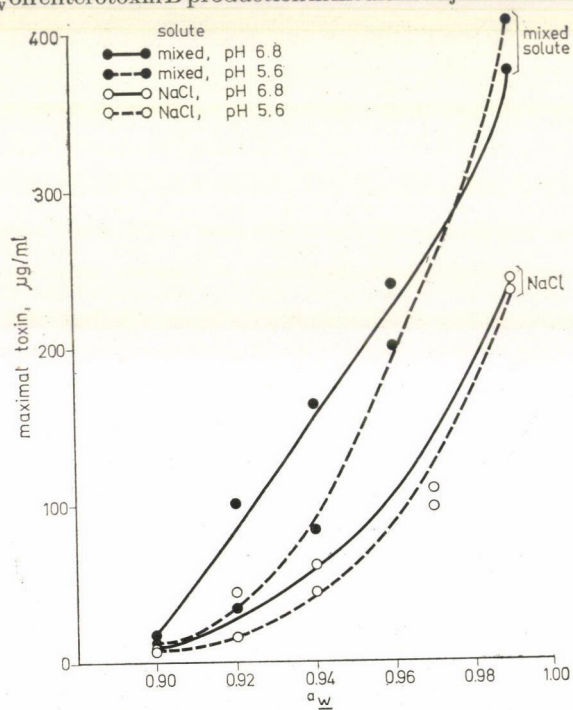


Fig. 6. Effect of  $a_w$  and pH on maximal enterotoxin *B* levels

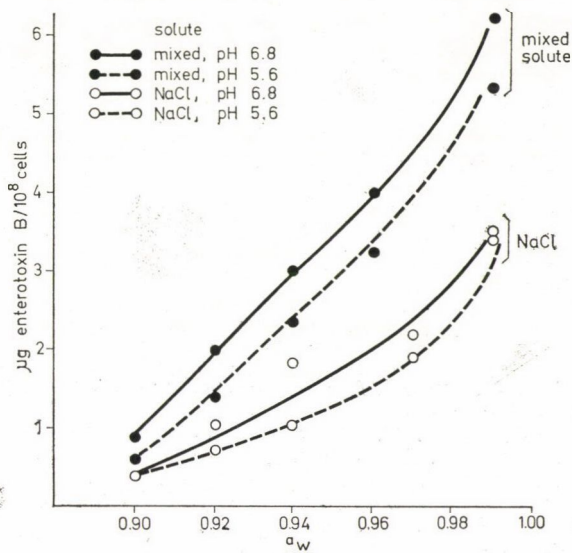


Fig. 7. Effect of  $a_w$  and pH on relative enterotoxin yields

### 3. Conclusions

Previously reported results from our laboratory (TROLLER, 1971) showed that  $a_w$  levels profoundly affected the production of enterotoxin B by *S. aureus* C-243.  $a_w$  reduction of 0.02 units from 0.99 or 0.98 to 0.97 or 0.96 reduced the ability of staphylococci to produce toxin by 90% or less of that occurring in high  $a_w$  control flasks. A subsequent report (TROLLER, 1972) on the effect of water activity on enterotoxin A corroborated these results, although the magnitude of the toxin suppression was somewhat less than for enterotoxin B. Unpublished studies on experimental foods, including shrimp slurries, have indicated that  $a_w$  limitation also reduces toxin levels in these systems.

The present study confirms previous reports and extends these results to a different strain of *S. aureus*. In addition, NaCl has been found to be significantly more suppressive to enterotoxin B production than a mixed solute system. The initial pH levels in combination with various water activities of the growth media do not appreciably affect the rate or amount of toxin produced or growth of the test organism. This is probably due to the rapid, upward readjustment of the pH in the medium during staphylococcal growth which would tend to negate the low initial pH levels.

The presence of solutes which tie-up or limit the amount of water available to enterotoxigenic staphylococci appears to limit severely the amount of toxin produced. The mechanism by which such solutes exert their effect is currently under investigation. CHRISTIAN and WALTHO (1962) describe the accumulation



of intracellular proline in staphylococci in limited water conditions. The conversion of other amino acids to proline could cause a depletion in the supply of various amino acids essential for enterotoxin synthesis and thus an apparent suppression of synthesis could occur. Studies on the parameters which govern the elaboration of toxins in foods, while few in number at present, are vitally needed to extend our understanding of techniques by which foods can be made safe and more wholesome for the consumer.

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## EFFECT OF NITRITE ON THE MICROBIOLOGICAL STABILITY OF CANNED VIENNA SAUSAGES PRESERVED BY MILD HEAT TREATMENT OR COMBINATIONS OF HEAT AND IRRADIATION\*

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Keeping quality of Vienna sausages packed in No. 1/5 cans (ca 200 g) with salt brine and heat treated with  $F_0$  values in the range of 0.25—0.55 was studied as a function of sodium nitrite addition.

In uninoculated cans the heat requirement of preservation proved to be about 0.3  $F_0$  in the presence of 300 ppm  $\text{NaNO}_2$ . A heat treatment of 0.55  $F_0$  and 200 ppm  $\text{NaNO}_2$  resulted in higher microbiological stability of cans inoculated with  $2 \times 10^4$ /tin *Clostridium sporogenes* spores than a heat treatment of 1.9  $F_0$  without nitrite addition.

The effect of the combination of heat treatment of 0.35—0.55  $F_0$  and 0.45 Mrad of gamma irradiation was also studied with inoculated cans. The combination of irradiation plus heat resulted in a higher microbiological stability of the samples than the reversed order of the treatments, but this synergistic effect could not be proved in all experiments.

The addition of 200 ppm  $\text{NaNO}_2$  was not as effective in increasing the shelf-life of combination treated samples as with the solely heat treated ones.

In a medium composed of a 50% extract of Vienna sausages (pH 6.4,  $a_w$  0.96) and inoculated with  $10^5$  per ml *Clostridium sporogenes* spores 100 ppm or more  $\text{NaNO}_2$  was required to ensure microbiological stability of samples heat treated with  $F_0$  0.4.

In the range of the permitted concentration level for canned meat products, sodium nitrite did not influence the heat resistance of *Clostridium sporogenes*, but inhibited the germination of the surviving spores.

Several investigations carried out in recent years drew attention to the observation that the surprisingly favourable statistics of food poisoning in relation to, and the microbiological stability of, preserved meat products exposed to comparatively low heat treatment may be due, at least partly, to the increased sensitivity to environmental factors of the bacterial spores surviving but damaged by heat treatment (RIEMANN, 1963; ROBERTS *et al.*, 1966). The transformation of heat damaged spores into vegetative cells is inhibited to a greater extent by the salt or nitrite content of the medium, than the transformation of untreated spores (ROBERTS & INGRAM, 1966; DUNCAN & FOSTER, 1968; FARKAS, 1969; DUNCAN, 1970; PIVNICK & THACKER, 1970a, 1970b; COOK & GILBERT, 1969; LABBE & DUNCAN, 1970).

Numerous data are also available on the increased sensitivity of irradiation damaged bacterial spores (KRABBENHOFT *et al.*, 1964; ROBERTS *et al.*, 1965; FARKAS, 1968, 1970; ROBERTS, 1970).

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However, in the majority of these investigations the behaviour of the damaged spores was studied in laboratory nutrient media. Therefore, in view of the practical requirements, it seems desirable to extend these investigations over foods to be preserved. As has been reported on in another paper at this symposium (INCZE *et al.*, 1973) favourable results were achieved with Vienna sausages canned in salt-nitrate containing brine and given mild heat treatment and medium radiation treatment, successively. If, however, the samples were inoculated with  $2 \times 10^4$  per tin *Clostridium sporogenes* spores, storage stability could not be achieved either by the combination treatment, or by heat treatment at  $F_0 = 2.8$ . Therefore we aimed at establishing the effect of nitrite upon the microbiological stability of canned Vienna sausages given mild heat treatment or a combination of heat and radiation treatment.

### 1. Materials and methods

Vienna sausages, canned in tins of about 200 g capacity, made of unlacquered tin plate and filled up with a brine containing 3% salt, 0.1%  $\text{KNO}_3$  and 0.2% tartaric acid were used as test material in a part of the experiments. The Vienna sausages were prepared in the pilot plant of the Hungarian Meat Research Institute with nitrite containing curing salt and without casing. The cans contained 3 sausages each and the sausage-brine ratio was approximately 1 : 1. During preparation, prior to peeling and canning, the sausages were smoked for 20 to 30 minutes at 80–90 °C and cooked for 200 minutes at 70 °C. The pH of the brine, independently of treatment, was 5.4–5.5. The equilibrium relative humidity of the product was 96% ( $a_w = 0.96$ ).

In order to study the effect of nitrite 400 or 600 ppm of  $\text{NaNO}_2$  was added to the brine in place of  $\text{KNO}_3$  and tartaric acid, thus the initial  $\text{NaNO}_2$  concentration (prior to treatment) in relation to the product was 200 or 300 ppm.

In other experiments the peeled sausages were comminuted immediately upon preparation and the mass thus obtained was macerated with identical quantity of 3% salt solution for 10 minutes at 40 °C. The solid particles were then allowed to settle and separated by centrifuging for 30 minutes and the supernatant (50% sausage extract) was used as test material. In aliquots of the extract definite amounts of  $\text{NaNO}_2$  were dissolved and then the extract was distributed in sterile test tubes, 9 ml each, under sterile conditions.

In some of the experiments the samples were not inoculated, however, the majority was inoculated with the spore suspension in 2.5%  $\text{NaCl}$  solution of a *Clostridium sporogenes* strain originating from the stock collection of the Central Food Research Institute. The suspension was prepared according to

the method of NATIONAL CANNERS ASSOCIATION RESEARCH LABORATORIES (1968).

When sausages were used in the experiments the spore suspension was inoculated in one of the sausages, prior to filling up the can with the brine. The spore suspension inoculated amounted to  $10^4$  spore concentration related to the content of the can. In the experiments carried out with 50% sausage extract the spore concentration was about  $10^5$ /ml.

After distribution of the sausage extract into the test tubes and inoculation the content of each test tube was covered with a 3-cm layer of sterile paraffin oil. The pH of these samples was 6.4, water activity  $a_w = 0.966$  and salt concentration 2.6%.

Equilibrium relative humidity was determined by the crystal liquefaction method of VAS and CSONTOS (1956) and salt content by the argentometric method of *Mohr*.

The canned sausages were heat treated in a steamer, the test tubes in a boiling water bath. The test tubes were immersed in the water bath deep enough to get the layer of paraffin oil below the level of the water. Heat penetration during treatment was measured with a thermoelectric thermometer manufactured by *Ellab*. The sterilization equivalent of the heat treatment ( $F_0$ ) was established by presuming  $z = 10^\circ\text{C}$ .

Irradiation was carried out with a  $^{60}\text{Co}$  gamma radiation source. On irradiating the canned sausages a dose rate of 0.3 Mrad/hour and for the 50% sausage extract 0.9 Mrad/hour was applied.

To establish nitrite concentration after treatment or during incubation of the samples the method of MÖHLER (1964) was used.

The samples (15 to 50 replicates per treatment) were stored in a thermostat at  $30^\circ\text{C}$ .

To establish viable cell count dilution technique of the most probable cell count was used in *Oxoid* Reinforced Clostridial Medium. Each sample was, after inoculation, covered with a layer of sterile paraffin. 5 parallel inoculations were made at each dilution level and the inoculated media were incubated at  $30^\circ\text{C}$ . To determine the cell count and to achieve heat activation prior to the preparation of the dilution series the 10-fold dilutions of the samples were treated for 20 minutes at  $80^\circ\text{C}$ .

## 2. Results

The spoilage percentage as a function of the logarithm of the heat treatment equivalent ( $F_0$  value), after a 5-week incubation period is shown in Fig. 1.

As is seen, the samples not inoculated and not containing added nitrite show storage stability if given a heat treatment of  $F_0 = 0.55$  sterilization



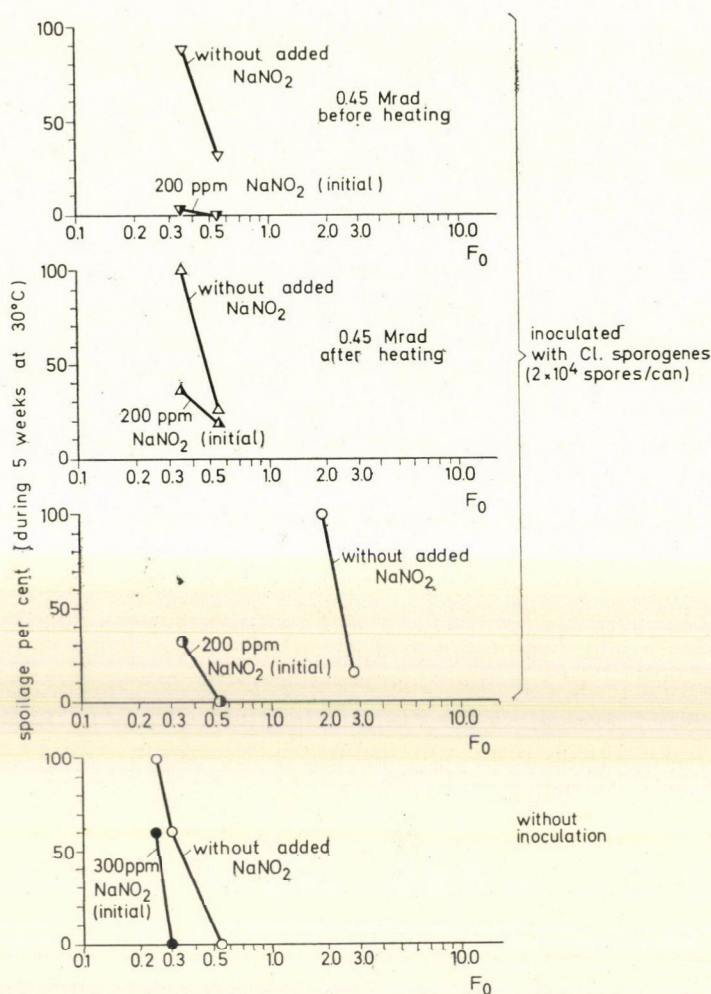


Fig. 1. The spoilage percentage of canned Vienna sausages as a function of the logarithm of the heat treatment equivalent ( $F_0$  value), after a 5-week incubation period at 30 °C

equivalent, but when given only  $F_0 = 0.3$  heat treatment 60% spoilage is observed after a 5 week storage period.

However, when 300 ppm  $\text{NaNO}_2$  was added before heat treatment, a treatment of  $F_0 = 0.3$  sterilization equivalent prevented spoilage. After heat treatment the average residual nitrite content in these samples was 130 ppm and after 9 days storage at 30 °C this value was reduced by 23–53%.

Complete microbiological stability of the samples inoculated with  $2 \cdot 10^4$  spores/can of *Clostridium sporogenes* could not be achieved even with a heat treatment of  $F_0 = 2.8$  sterilization equivalent, if the brine contained salt and nitrate in the traditional way. On the other hand samples containing

200 ppm  $\text{NaNO}_2$  prior to heat treatment did not show spoilage during an incubation period of 5 weeks when given a heat treatment of only  $F_0 = 0.55$ .

The successive application of a heat treatment of  $F_0 = 0.55$  and irradiation with 0.45 Mrad the stability of the samples approximated, during the first five weeks of incubation, that of the samples given a heat treatment of  $F_0 = 2.8$  sterilization equivalent, without irradiation.

Addition, before combined treatment, of 200 ppm  $\text{NaNO}_2$  did not increase the microbiological stability to the same extent as in samples given only heat treatment, though spoilage was lower in samples containing nitrite than in samples without added nitrite. Also the order of the combined treatments affected the stability of the samples. Out of four experimental batches irradiated first and then heat treated the stability of three was higher than that of samples treated in reversed order. The efficiency of nitrite addition and combination treatments can also be evaluated by comparing the respective  $F_0$  values required to ensure the microbiological stability of two thirds of the parallel samples (Table 1).

The experiments carried out with Vienna sausages inoculated with *Clostridium sporogenes* spores were extended to the effect of the reduction of water activity to the extent not causing unacceptable organoleptic quality, upon the efficiency of the combination treatment. The reduction of water activity seemed expedient on the basis of the investigations of LEISTNER and KARAN-DJURDJIC (1970). They found increased storage stability in preserves with a water activity reduced to 0.94.

According to preliminary experiments the salt concentration cannot be substantially increased without decreasing the sensory quality of canned Vienna sausages. Therefore water activity was reduced with a combination of

Table 1

*Heat treatment requirement of canned Vienna sausages to achieve microbiological stability in 67% of the samples inoculated with  $2 \cdot 10^4$  Clostridium sporogenes spores/can at  $a_w = 0.96$  and 5 weeks storage at 30 °C*

Treatment	Heat treatment requirement $F_0$
Brine containing no $\text{NaNO}_2$	2.6
200 ppm initial nitrite content	0.35
Irradiation with 0.45 Mrad after heat treatment, $\text{NaNO}_2$ not added	0.53
Irradiation with 0.45 Mrad prior to heat treatment, $\text{NaNO}_2$ not added	0.55
Irradiation with 0.45 Mrad after heat treatment and 200 ppm initial $\text{NaNO}_2$ concentration	0.38
Irradiation with 0.45 Mrad prior to heat treatment and 200 ppm initial $\text{NaNO}_2$ concentration	0.35



NaCl and glycerol which in the preliminary experiments had no detrimental effect on the organoleptic value of the product. A water activity of 0.94 was achieved by 4% NaCl and 3% glycerol concentration related to the brine. Samples were then given a heat treatment of  $F_0 = 0.55$  sterilization equivalent and irradiated with 0.45 Mrad. Samples containing no added nitrite and not irradiated served as control.

The spoilage curves of the experimental batches as established during the incubation period at 30 °C are shown in Fig. 2.

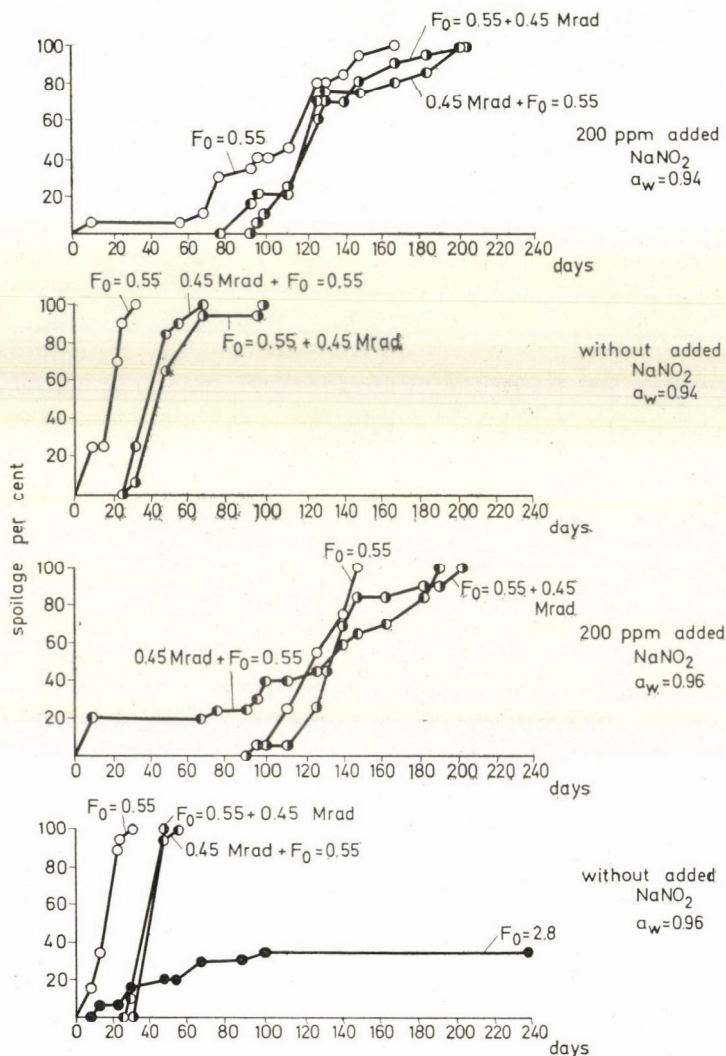


Fig. 2. The spoilage curve of the experimental batches of canned Vienna sausage during the incubation period at 30 °C

To evaluate these curves their probit transformation was carried out and from the correlation of the probit values versus storage time graphs the average spoilage times (spoilage half-time,  $\bar{t}$ ) and the standard deviations were determined. These are illustrated in Fig. 3.

On the basis of the  $\bar{t}$  values the brine and physical treatment-variants were subjected to analysis of variance.

Analysis of variance permits of the conclusion that the addition of nitrite substantially increased the half-time of spoilage. However, reduction of water activity from 0.96 to 0.94 by adding a combination of salt and glycerol to the samples caused no improvement in storage stability. On the contrary, in samples containing nitrite the half-time of spoilage was somewhat reduced by 4%

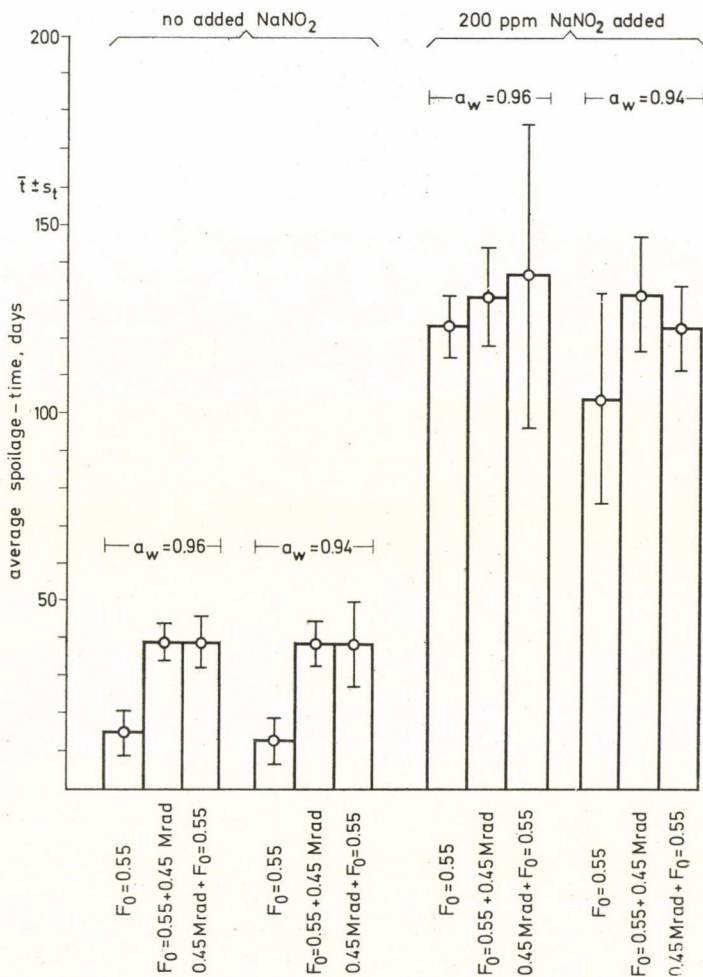


Fig. 3. The spoilage half-time values and the standard deviations as a function of treatment and water activity



NaCl and 3% glycerol. As is obvious, the combination of irradiation with heat treatment increased storage stability with all the brines. It is worth noting that the effect of nitrite was weaker in the samples given combination treatment than in samples only heat treated. In the case of heat treated samples the half-time of spoilage of the nitrite containing samples was more than 8 fold of those not containing nitrite, while with samples subjected to combination treatment this ratio was only about 3.5. Statistical analysis showed the order of combined treatments not to affect significantly their efficiency in this series of experiments.

Another observation worth mentioning was that in the initial stage of spoilage of the samples containing added nitrite, according to tests carried out on the 90th day of incubation, the nitrite content of samples not yet spoiled was 6–9 ppm, whereas that of the spoiled samples 3–5 ppm.

Experiments carried out with sausage extract were aimed at the study of the effect of reduction of nitrite concentration. In these experiments, taking into account practical requirements and public health aspects (BERESINA & CHOLODNOWA, 1968; LIJINSKY, 1971; ANON, 1971b; MIRNA, 1972) higher initial concentrations than 250 ppm  $\text{NaNO}_2$ , were not applied.

Fig. 4 shows the results of an experiment in which 50% sausage extract samples, inoculated with  $10^5/\text{ml}$  *Clostridium sporogenes* spores, not heat activated, and containing various amounts of  $\text{NaNO}_2$  were exposed to heat treatment of  $F_0 = 0.4$  sterilization equivalent (60 minutes in a water bath of 100 °C). The figure shows the spoilage percentage of the samples after a 5-week incubation period at 30 °C, and the  $\text{NaNO}_2$  concentrations prior to heat treatment and after heat treatment.

As it is seen in the figure, a  $\text{NaNO}_2$  concentration of 190 ppm after heat treatment gave complete storage stability under the prevailing conditions. At 120 ppm initial  $\text{NaNO}_2$  concentration corresponding to 85 ppm  $\text{NaNO}_2$  after heat treatment, only 7.5% of the samples underwent spoilage. At 50 ppm initial  $\text{NaNO}_2$  concentration 94% of the parallel samples underwent spoilage and at 30 ppm initial  $\text{NaNO}_2$  concentration 80%.

Plotting the quantal spoilage ratio as a function of residual nitrite concentration the  $\text{ED}_{50}$  value of nitrite, or the quantity of residual nitrite, inhibiting spoilage in 50% of the parallel samples, could be estimated.

This  $\text{ED}_{50}$  value was found to be about 60 ppm. Upon storage for nearly three months the residual nitrite concentration and anaerobic viable cell count in the unspoiled samples showed only a variation characteristic of the test methods and the inhomogeneity of the samples, thus these might be considered as not significantly altered. Fig. 5 illustrates the anaerobic viable cell counts of samples initially containing 200 ppm  $\text{NaNO}_2$  as determined in samples not heat activated and heat activated for 20 minutes at 80°, respectively.

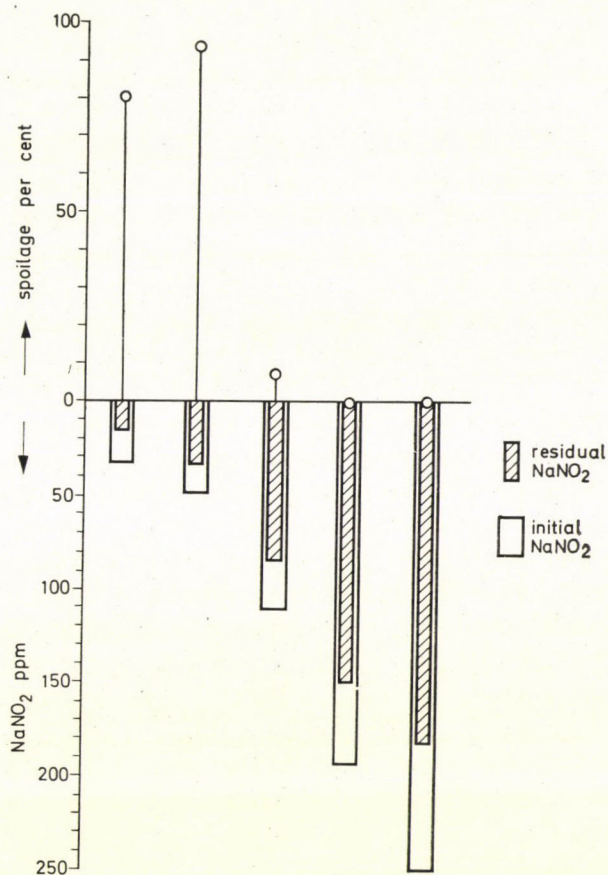


Fig. 4. The effect of added  $\text{NaNO}_2$  on the spoilage of 50% sausage extract samples, inoculated with  $10^5/\text{ml}$  *Clostridium sporogenes* spores and exposed to heat treatment of  $F_0$  0.4 sterilization equivalent. The figure shows the spoilage percentage of the samples after a 5-week incubation period at  $30^\circ\text{C}$  and the  $\text{NaNO}_2$  concentration prior to heat treatment and after heat treatment

The residual concentration of  $\text{NaNO}_2$  of about 150–170 ppm means that about 75 to 85% of the added nitrite could be detected in the 50% sausage extract after a heat treatment of  $F_0 = 0.4$ . [For the sake of comparison it is interesting to note that after a heat treatment of  $F_0 = 0.8$  PIVNICK and co-workers (1970a) detected about 46–66% of the added  $\text{NaNO}_2$  in the 50% meat extract.]



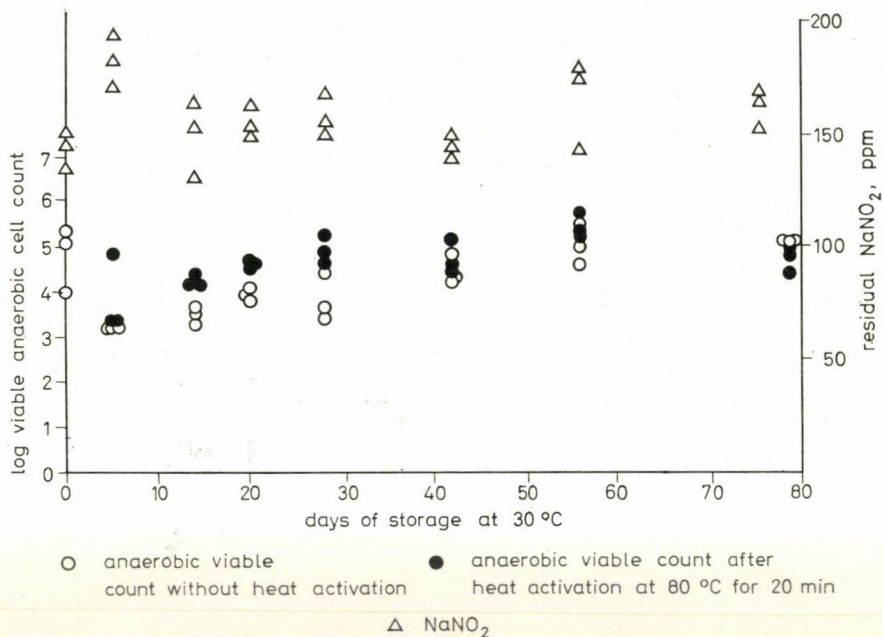


Fig. 5. The anaerobic viable cell counts and residual  $\text{NaNO}_2$  contents of 50% sausage extract samples heat treated with  $F_0$  0.4 sterilization equivalent as a function of storage time

### 3. Conclusions

It is of interest that the residual nitrite concentration in the sausage extract contained in test tubes was not reduced even during a longer storage period, in contrast to the nitrite content of canned sausages as described above. On the basis of data found in the literature (NORDIN, 1969; ASHWORTH & SPENCER, 1972) a 5-day half-life on nitrite can be reckoned with. These contradictions may be explained, on one hand, by the metal of the unlaquered tin cans promoting the decrease in  $\text{NaNO}_2$ , and on the other, by the circumstance that cured meat used in the experiments does not react with the subsequently added nitrite in the same way as meat not cured.

On evaluating the results of our experiments it is worth noting that in samples not inoculated relatively very low  $F_0$  values are sufficient to provide complete storage stability of the samples. This observation is in good agreement with data found in the literature. According to HEIDTMANN and REICHERT (1969)  $F_0$  values of 0.6–0.8 ensure good stability in canned Vienna sausages. Since, however, the survival of microbial spores, particularly those of *Clostridium* strains causing food poisoning, is not prevented by such low heat treatment, the factors inhibiting surviving spores are of great importance (LEISTNER

et al., 1970; DUNCAN, 1970). Beside storage temperature, which was not investigated in this study, the role played by curing salts and water activity is of great importance. From the behaviour of samples prepared with traditional nitrate containing brine it may be concluded that nitrate at the concentrations applied (500–1 000 ppm) has no specific effect upon *Clostridium sporogenes* spores and beside sodium chloride in much higher concentrations, the permissible nitrate level does not significantly affect water activity either. These findings are in good agreement with the results of other similar experiments (SILLIKER et al., 1958; DUNCAN, 1970; ANON, 1972b).

The important role of salt concentration or water activity in the propagation of microorganisms is common knowledge. In experiments carried out with other products (FARKAS, 1971)  $a_w = 0.94$  value set with NaCl was found to have a sporostatic effect. To achieve this water activity in Vienna sausages a salt concentration inacceptably high from the organoleptic point of view is necessary. The combination of glycerol and NaCl as applied in our experiments to reduce  $a_w = 0.96$  to  $a_w = 0.94$  just to overcome the above difficulty did not cause increased inhibition of spores, on the contrary, it somewhat reduced the efficiency of mild heat treatment or of irradiation combined with heat treatment. In interpreting the results of the present experiments we consider probable the dependence on water activity to be proportional to the extent of damage in the spores, and the mild heat treatment as applied in the experiments caused no severe damage in the spores. This is supported by viable cell count determinations, here not described in detail, showing that practically no reduction of viable spore count occurred during the heat treatment. Probably the results obtained by water activity reduction with a glycerol-NaCl mixture indicate a specific inhibiting effect of NaCl which property is not characteristic of glycerol. Thus an  $a_w = 0.94$  value set with a glycerol-NaCl mixture is not equivalent to  $a_w = 0.94$  set with NaCl only. Several authors (BAIRD-PARKER & FREAME, 1967; KANG et al., 1969; STRONG et al., 1970; JAKOBSEN et al., 1972) suggest that at identical water activity values NaCl has a stronger bacteriostatic effect than polyhydroxi-compounds. The possibility of increased heat and radiation tolerance in spores of reduced water activity cannot be precluded either.

The experiments proved unambiguously the effective inhibition of spoilage by the addition of  $\text{NaNO}_2$  above 100 ppm. It was shown by the investigations that the addition of 150–200 ppm  $\text{NaNO}_2$ , as permitted by the public health authorities of different countries (FEDERAL REGISTER, 1968; RICHARDSON, 1970; ANON, 1971a) does not reduce the heat tolerance in *Clostridium sporogenes* spores but inhibits the outgrowth of surviving spores.

In samples exposed to irradiation combined with heat treatment nitrite was less effective than in samples heat treated only, probably because irradiation helped to reduce the initial nitrite concentration.



KEMPE and GRAIKOSKI (1964) reported on similar observations. On irradiating luncheon meat they found that the *Clostridium botulinum* inhibiting effect of the curing salts was reduced by radiation treatment.

The role played by nitrite in the storage stability of cured meat products is not unknown, but rather contradictory. Though already in 1929 GRINDLEY attributed a bacterium inhibitory effect to nitric acid, a breakdown product of nitrate (GRINDLEY, 1929), the inhibitory effect of nitrite was proven much later by TARR (1941a, b, 1942). The first paper discussing the mechanism of bacterium inhibition was published in 1954: JENSEN (1954) attributed this effect to undissociated nitric acid. This finding was later confirmed by several authors (EDDY & INGRAM, 1956; PERIGO *et al.*, 1967) giving at the same time a satisfactory explanation to the role of pH, too (HENRY *et al.*, 1954; ROBERTS & INGRAM, 1966). With decreasing pH increases the number of undissociated molecules intensifying thereby the inhibitory effect of nitrite (INGRAM *et al.*, 1956; MOL & TIMMERS, 1970). The inhibitory effect increases up to pH = 4.5—5.5, since below this value nitrite becomes rapidly inactivated, probably due to the van Slyke reaction. The investigations proving that the inhibitory effect of nitrite can be intensified by ascorbic acid (HENRY *et al.*, 1954; SIMONSEN, personal communication) and by autoclaved glucose, show that beside pH also the reduction of the redox potential partakes in this inhibitory procedure (EDDY & INGRAM, 1956; CASTELLANI & NIVEN, 1955).

A new age was opened in the study of the effect of nitrite when the effect after heat treatment was investigated (STEINKE & FOSTER, 1951). BULMAN and AYRES (1952) observed that in spite of relatively low heat treatment (20 minutes at 80 °C) the growth of PA 3679 spores can be inhibited and the spoilage of minced meat prevented by adding 800 ppm nitrite, when 50 spores/g were inoculated. After the breakdown of the nitrite during storage (to 4 ppm) spoilage ensued.

SILLIKER and co-workers (1958) inoculated cured meat trimmings with 2.6 PA 3679 spores/g and found 78 ppm nitrite to prevent spoilage when the sample was heat treated at  $F_0 = 0.1$ . At higher cell count, or identical cell count but applying NaCl instead of nitrite, spoilage ensued.

The results obtained in our experiments confirmed data published earlier (RIEMANN, 1963) showing that viable spores could be isolated from nitrite containing stable meat products. Nitrite has probably no heat sensitizing effect but by inhibiting outgrowth or/and germination of spores ensures the stability of the product (ROBERTS & INGRAM, 1965; SPENCER, 1966).

An interesting turning point was brought about in the investigation of the reaction mechanism of nitrite by the publication of PERIGO and co-workers (1967). Using *Clostridium sporogenes* as test organism, they achieved the same spore inhibiting effect in microbiological medium with 3—5 ppm nitrite when given a heat treatment of 20 minutes at 105—115 °C as with 200—400 ppm



nitrite not heat treated. The 3–5 ppm nitrite concentration was more or less independent of the pH of the medium. In further studies the same inhibitor (PERIGO & ROBERTS, 1968) proved very effective against *Clostridia*, *Clostridium botulinum* as well.

When similar experiments were carried out with meat (DUNCAN & FOSTER, 1968; JOHNSTON *et al.*, 1969; LABBE & DUNCAN, 1970; SIMONSEN, 1970; SPENCER, 1971) from the interaction of meat and nitrite, at least in the range of permitted nitrite addition, the substance of high inhibitory effect, which developed when nitrite reacted with a component of the nutrient medium, was not formed and the inhibitor formed in the nutrient medium became inactivated when added to meat. Trials to separate the inhibitor by column chromatography were not successful either (ROBERTS, 1971).

On the detection of the PERIGO effect in meat ASHWORTH and SPENCER reported only recently (1972). They succeeded in demonstrating that nitrite concentration exceeded 300 ppm prior to heat treatment. However, the increase in the efficiency of the inhibitor thus formed was not as high as that observed by PERIGO and co-workers (1967) on the conjugate heat treatment of nitrite and medium. However, these authors used untreated test organisms to inoculate the heat treated medium, therefore from their experiments conclusion as to the behaviour of microorganisms surviving and damaged by heat treatment, or heat treated in the presence of nitrite, cannot be drawn. Thus the role of the PERIGO effect in the microbiological stability of meat preserves prepared from cured meat is not yet elucidated. This problem naturally does not bear upon the spore inhibitory effect of inorganic nitrite.

In our experiments a residual nitrite concentration of about 100–150 ppm ensured good storage stability to canned Vienna sausages or 50% sausage extract, given low heat treatment, even under unfavourable pH conditions (pH = 6.2–6.4) and an inoculum of high cell concentration. This fact, in accordance with other research work (ANON, 1972a) shows the importance of nitrite in the stability and microbiological safety of canned meat products prepared of cured meat and given a low heat treatment, and in the prevention of botulism, whether it is due to the PERIGO effect or to inorganic nitrite. Thus in preserves, where the reduction of the water activity below  $a_w = 0.95$  is not possible without depreciating sensory quality, the reduction of the permissible nitrite concentration, from 150–200 ppm to 10–30 ppm as suggested by certain authors (BERESINA & CHOLODNOWA, 1968; LIJINSKY, 1971), might raise problems in view of the microbiological safety and stability of the products.



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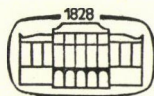
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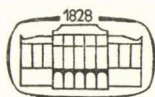
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## INACTIVATION OF SPORES IN FOOD BY COMBINED HEAT AND HYDROSTATIC PRESSURE\*

G. W. GOULD

(Received September 22, 1972)

Bacterial endospores are normally extremely resistant to high hydrostatic pressures: spores of many organisms will survive exposure for many hours at pressures in excess of 8 000 atmospheres. However, under certain conditions pressures as low as 100 atmospheres will initiate the germination of bacterial spores, thus sensitizing them to heat, radiation and chemical agents.

Initiation of germination by pressure is highly temperature-dependent, being generally increased with increase in temperature, although in the lower pressure range (hundreds of atmospheres) well defined temperature optima are recognisable. "Pressure germination" is affected by pH and ionic environment in such a way as to suggest that spore metabolism must be involved rather than solely the physical distortion of the spore.

Those spores that are the most resistant to heat are not necessarily also resistant to pressure. However, those spores that are the most dormant and difficult to cause to germinate (*i.e.* with nutrients) at ambient pressure are also the most reluctant to germinate under increased pressure.

Hydrostatic pressure can be used to reduce the numbers of spores in food-stuffs, most effectively if combined with a mild (pasteurizing) heat treatment or with irradiation. However, the effectiveness of pressure is severely limited by the extreme dormancy of many naturally occurring spores and by the small fraction of "superdormant" spores that occur in all spore populations.

It is widely accepted that the endospores of some bacteria and thermophilic actinomycetes represent the living forms that are most resistant to a wide range of environmental stresses; including, for example, resistance to desiccation, wet and dry heat, ionizing and ultraviolet radiations, acids, alkalies, disinfectants and antibiotics (ROBERTS & HITCHINS, 1969). Consequently, the observation, reported more than half a century ago by LARSON *et al.* (1918), that bacterial spores also resisted extreme hydrostatic pressures was not considered surprising, even though the pressures of up to about 12 000 atmospheres (atm) used by these research workers and later by BASSET and MACHEBOEUF (1932) were well in excess of the pressures which can directly denature native proteins *in vitro* and *in vivo*, and well in excess of the pressures that are rapidly lethal for vegetative forms of bacteria, *e.g.* 1 000—3 000 atm (HITE, 1899; HEDÉN, 1964).

\* Presented at the IUFoST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



Recently, however, CLOUSTON and WILLS (1969) and SALE *et al.* (1970) re-examined the effects of hydrostatic pressure on bacterial spores and whilst confirming that spores certainly can resist the high pressures reported by the earlier workers, nevertheless, found that under certain conditions much lower pressures could lead to the inactivation, or killing, of spores.

The most unexpected observation that came out of this work was that spores could in fact be inactivated more effectively by *low* than by *high* pressures under some conditions. This unexpected observation stimulated further research, firstly in an effort to understand the mechanisms of inactivation and spore resistance and secondly to evaluate the feasibility of using pressure as an aid to food preservation.

### 1. Materials and methods

Samples of bacteria in suspensions or of foods were sealed into small polythene sachets. These were immersed in oil in conventional pressure intensifiers which were run off high pressure hydraulic pumps. The pressure vessels could be brought up to 8 000 atm in about 30 sec and lowered in about 5 sec. Care was taken to avoid heat effects, for the adiabatic heating of samples amounted to about 3°C per 1 000 atm; the temperature transient decayed away in about 10 min. The vessels contained thermocouples, so that temperature near the sachets was monitored, and were jacketed so that the pressurized samples could be thermostated at any temperature up to 100°C.

Spores were prepared and enumerated as described previously (SALE *et al.*, 1970).

### 2. Results

Early work on pressure-inactivation of spores was mostly concerned with total inactivation of populations, and for this reason interesting fractional effects were overlooked.

The general pattern of response of spores to pressure that we found was in fact complex and highly dependent not only on the pressure but also on other environmental parameters, in particular the temperature.

Table 1 summarises the response of spores to pressure, firstly before pasteurization — and secondly after a post-pressurization pasteurization. There is evidently a large temperature coefficient, which was quantitatively different, but qualitatively similar for spores of other *Bacillus* and *Clostridium* species.

In general, up to about 1 000 atm, inactivation increased with pressure; between about 2 000 and 3 000 atm inactivation tended to level off; inactivation increased again as the pressure was raised further; above about 4 000

atm inactivation continued to increase if the temperature was above about 55°C, but decreased with increase in pressure if the temperature was below this.

Comparison of the data before and after pasteurization at 70° for 30 min suggested that pressure caused heat-sensitization of a fraction of the surviving population.

Table 1

*Inactivation of spores of Bacillus coagulans by hydrostatic pressure at different temperatures*

Temperature of pressurization	Survivors (%) following pressurization (30 min; pH 8.0) at (atm)			
	2 000	4 000	6 000	8 000
<i>Before pasteurization*</i>				
25°	12	16	85	90
45°	2	0.12	3.3	11
55°	0.08	0.001	0.0007	0.002
65°	0.007	<0.00001	<0.00001	<0.00001
<i>After pasteurization*</i>				
25°	4	3	13	88
45°	0.2	0.00001	0.00001	0.00008
55°	0.03	<0.00001	<0.00001	<0.00001
65°	0.0002	<0.00001	<0.00001	<0.00001

\* Pasteurization was at 70° for 30 min.

Associated changes occurred which were all typical of germination — *e.g.* calcium, dipicolinic acid and hexosamine-containing peptides were excreted, the spores became stainable and phase-dark, and in electron micrographs of sectioned spores it was seen that pressure caused a loss of the cortex. CLOUSTON and WILLS (1969) reported that pressure caused an increase in spore radiation sensitivity.

In fact, pressure seemed to induce, even in water with no added nutrients, what appeared to be typical germination: that is, germination just like that normally caused by nutrient germinants: and at least at the *lower* temperatures, low pressures were more germinative than higher pressures.

Looked at another way, the effect of pressure was to *cause* germination and also to raise the optimum temperature for germination. This trend is well known with isolated enzyme reactions also, where instances are known of enzymes for example optimally active at temperatures as high as 101°C under increased pressure. Moderate pressures tend to arrest the first small molecular volume increases associated with thermal denaturation, whils



higher pressures of the order of thousands of atm will actually cause denaturation by inducing an irreversible reduction in molecular volume.

pH value affected pressure germination in a similar manner to germination initiated by more conventional nutrient germinants except that the pH limits were wider. The observation that extreme pH values inhibit pressure germination is very significant in that it suggests that pressure germination might involve metabolism rather than solely result from physical forces on the spore. Physical forces would be unlikely to be interfered with seriously by pH, and even more unlikely would be a physical effect interfered with by both acid and alkali. The picture is more like that which one would expect to see from physiological or enzymic reactions.

Involvement of spore metabolism was further suggested by the effect of solutes. Non-ionic solutes hardly affected pressure germination, whilst ionic solutes were inhibitory (Table 2), somewhat like the normal or "physiological" germination response of spores.

The final indication that metabolism was implicated in pressure germination was that various inhibitors of normal germination also inhibited germination by pressure. However, it was true that as the pressure was raised, the inhibitory effects of these substances became less and less, as did the relative effects of extreme pH values and of high concentrations of salts mentioned above.

Table 2  
*Inhibition of pressure — induced germination by solutes*

Solute	Ungerminated spores (%) following pressurization (30 min; 30°C; pH 8.0) in solutions of water activity				
	1.00	0.99	0.98	0.96	0.94
Sodium chloride	0.01	0.1	0.4	5.7	8.2
Glycerol	0.01	0.06	0.05	0.04	0.05

So far we have considered the germinative effects of pressure acting on spores in water or in buffer solutions. When we investigated the interaction of pressure with more conventional, or 'nutrient' germinants, we found a strong synergism (Table 3). Just as the *relative* effect of inhibitors became less and less as the pressure was raised, so the relative effect of the germinants became less at high pressures.

A change in specificity of spores for germinants under pressure was shown for L- and D-isomers of alanine. The L-form is normally germinative and the D-form is inhibitory at 1 atm. But under moderate pressure, even D-alanine became germinative. We used O-carbamyl-D-serine (OCDS), which inhibits racemization of alanine by spore alanine racemase, to find the reason



for this unusual action of D-alanine. It appeared that pressure speeded up racemization so that L-alanine was quickly formed from the D-isomer and that, in addition, the D-isomer was less inhibitory under pressure than at 1 atm.

With spores of *Bacillus* and of *Clostridium* species pressure caused a similar germination both aerobically and anaerobically. Spore concentration did not affect the percentage germination or inactivation over the range  $10^5$  to  $10^9$  spores per ml.

Table 3

*Synergistic action of hydrostatic pressure with physiological germinants*

Germinant (1 mM)	Germination (%) following pressurization (30 min; 30°C; pH 8.0) at (atm.)		
	Unpressurized control	200	1 000
No addition	0	0	99.6
L-Alanine	45	80.5	99.8
L-Glutamine	47	88	—
L-Cysteine	0	96	—

The pressurization rate and the depressurization rate were unimportant in determining the amount of germination. Multiple pressurizations were no more effective than a single pressurization for the same total time. The amount of germination was *essentially a function of holding time under pressure*.

Pressure would effectively germinate spores in food samples and therefore reduce the contaminating heat resistant spore load in a food. A great advantage in the use of pressure in this way is the fact that hydrostatic pressure is not vectorial: that is, all parts of a food packed, for instance, in a flexible plastic pack, are equally treated when exposed to increased pressure; in contrast to heat, with problems of conduction, and radiation with problems, for instance, of geometry and penetration.

The effectiveness of pressure on spores in foods is illustrated by the reduction in numbers of spores of *B. cereus* and *B. polymyxa* in minced meat held at 2 000 atm 60°C for 1 hour. Reductions obtained were — for *B. cereus*  $10^5$ -fold and for *B. polymyxa*,  $10^6$ -fold. Less extensive reductions of numbers of other organisms were noticed, including *B. subtilis*, and *Cl. sporogenes*. Spores of *Cl. botulinum* type A were of average pressure sensitivity. A treatment of 2 500 atm at 70° for 1 hour was necessary to cause a reduction of about  $10^6$ -fold of *Cl. botulinum* spores in a rich medium.

The kinetics of germination of spores under pressure was complex. CLOUSTON and WILLS (1970) found inactivation to be a consecutive 1st order process for *B. pumilis* spores pressurized up to 1 100 atm in potassium phos-



phate at 25°C. We found various organisms to respond differently except that small fractions of every spore population were always more pressure-resistant than the majority of the population (GOULD & SALE, 1970). This phenomenon is very reminiscent of normal germination at normal pressures. Typical germination curves invariably plateau, in contrast to classical heat or radiation inactivation curves. The survivor in the 'tail' of a germination curve can be separated and shown to be truly more dormant to nutrient germinants than the bulk of the population.

Unfortunately, these 'superdormant' spores, which probably resemble naturally occurring spores rather more closely than the spores that most researchers use in their laboratories, are also dormant towards germination by pressure. It is, in fact, these more dormant, or superdormant (and probably more natural) spores that limit the usefulness of pressure as a preservation aid. Whilst laboratory spores like *B. cereus* are highly pressure sensitive, the more dormant cells that abound in nature, spores from soil, dust and air, are only inactivated for instance by  $10^1$ - or  $10^2$ -fold by pressures in excess of 3 000 atm at temperatures above 70°C. These conditions exceed the pressures that could readily and sensibly be used in practical food preservation systems.

### 3. Conclusions

High hydrostatic pressures, of about 100 atm and upwards, will cause spores to germinate. The effectiveness of pressure as a germinant is very much influenced by temperature and less so by other environmental factors, like pH, salts, nutrients and metabolic inhibitors. The mechanism of action of pressure has not been discussed in this paper: it is most likely that the initial action is to cause a rise in the free ion level in the spore caused by the electrostrictive effects of pressure (TIMSON & SHORT, 1965), and that this rise in ion level initiates enzyme activity which leads to depolymerization of spore components which are concerned with dormancy and resistance.

Unfortunately, naturally occurring spores vary widely in their dormancy and therefore also in their sensitivity to pressure. The extreme resistance of some of these natural spores is sufficient to seriously limit the usefulness of pressure as a preservation technique at our present state of knowledge.

Nevertheless, it is true to say that hydrostatic pressure combined with mild heating offers, for the first time for many years, a completely new approach to food preservation. It is not a viable approach at the present time because of the limitations mentioned above. However, with new understanding of the factors controlling dormancy of spores, these limitations might well be overcome in the future.

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## HEAT AND pH EFFECT ON MICROORGANISMS, CAUSING SPOILAGE OF CANNED FOODS\*

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The paper deals with the correlation of *Bac. stearothermophilus* 80 and *Cl. sporogenes* 25 spore thermoresistance in canned food and the pH value of food and shows that it can be expressed as an equation of a straight line.

It reveals that when heated the *Cl. pasteurianum* 148 spore population consists of thermolabile and thermostable cells. Thermoresistance of thermolabile cells at pH values in the range of 4.5 to 7.0 is independent of pH, while the thermoresistance of thermostable cells under similar conditions depends on hydrogen ion concentration.

It was found that the character of the *Cl. botulinum* survival curve varied with food pH value. At pH 5.0 the *Cl. botulinum* survival curve was expressed in a semilogarithmic co-ordinate system as a straight line, and at pH 4.5 as a curve, reflecting the probability distribution of spores according to the thermoresistance.

Thermoresistance of bacterial spores depends on heredity, physiological state of spores and conditions under which they exist during the period of heating. Concentration of hydrogen ions, expressed by the pH value, is one of the main factors, affecting spores during the heating period. Data on the effect of pH of the medium on thermoresistance of bacillus and clostridium spores are given in the literature in the form of equations (MAZOKHINA *et al.*, in press; MAZOKHINA *et al.*, 1971), tables (XEZONES & HUTCHINGS, 1965) and reports on certain experimental results. But most of the data have been obtained in experiments where buffer solutions or laboratory culture media were used. The purpose of this work was to find out the pH effect on thermoresistance of spore microflora, causing the spoilage of canned food in conformity with the formulae of heat treatment of canned fruit and vegetables manufactured in the USSR.

### 1. Materials and methods

*Bac. stearothermophilus* 80, *Cl. botulinum* B-4, *Cl. sporogenes* 25, *Cl. pasteurianum* 148 cultures, isolated from spoiled canned food have been used in the experiment. Spores of these microorganisms have been obtained by using generally adopted methods (ROGACHEV *et al.*, 1968), washed clean in a phosphate

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buffer solution of pH 6.9 by centrifugation and stored under 4°C before use. Canned food has been prepared according to commercial technology; it has been infected with spores to a level of  $10^6$  spores of *Bac. stearothermophilus* 80 and  $10^4$  spores of *Cl. sporogenes* 25 per 1 g of food at the moment the inside temperature of the can rose to the desired level. The heating of canned food infected with *Bac. stearothermophilus* 80 or *Cl. sporogenes* 25 has been carried out in 15-ml test cans in a laboratory autoclave, with a device for instantaneous temperature rise and fall up to the predetermined level and thermocouples for temperature registration in the autoclave and in the cans. Not less than 5 cans have been heated at each temperature-time combination.

*Cl. botulinum* B-4 and *Cl. pasteurianum* 148 spores have been heated in food or in buffer mixtures in capillaries.

After heating and inoculating into agar potato medium, the number of surviving *Bac. stearothermophilus* 80 has been determined and survival curves have been plotted in the semilogarithmic co-ordinate system, from which  $D_T$  values have been obtained graphically. The whole amount of the canned food, infected with *Cl. sporogenes* 25 spores after heating has been inoculated into bottles containing casein medium and  $D_{T_0}$  values have been calculated according to Schmidt's method (SCHMIDT, 1957). The thermoresistance of *Cl. pasteurianum* 148 and *Cl. botulinum* B-4 spores has been determined from the survival curves, by measuring the number of survivors using agar casein medium in high tubes.

## 2. Results

The effect of heating and that of the pH of the medium on *Bac. stearothermophilus* 80 spores has been studied using the following canned foods: mashed carrots with rice (pH = 5.5), mashed pumpkin with rice (pH = 5.8), mashed vegetable marrow with milk (pH = 5.8), meat and vegetable thick soup with tomato (pH = 5.2) and mashed liver with rice (pH = 5.85).

The dependence of thermoresistance of *Bac. stearothermophilus* 80 spores, expressed as  $D_{121^\circ}$  values, on the pH of food can be described by the following equation:  $D_{121^\circ} = 1.7 \text{ pH} - 7.5$ . The equation shows that even an 0.1 unit change in pH causes almost 20 per cent change in  $D_{121^\circ}$ .

The possibility of applying the results obtained to determine the duration of heat treatment of commercial canned baby food has been examined.

pH variations in food of different batches have been studied at several plants. The data obtained have been subjected to mathematical treatment. That is combined control-analytical cards of average pH values ( $\bar{X}_{\text{pH}}$ ) for the sample and their range ( $R_{\text{pH}}$ ) have been developed. A combined control card of average pH values and their range, when controlling the production of canned "Meat and vegetable thick soup" is shown in Fig. 1.

Average pH values of many batches exceeded the limits of  $\bar{X} \pm 3\sigma$  values, thus the sterilizing time can not be determined by using the average pH value of the food; it is necessary to determine it for each batch, or when calculating sterilization formulae, guaranteeing the safety against flat-sour

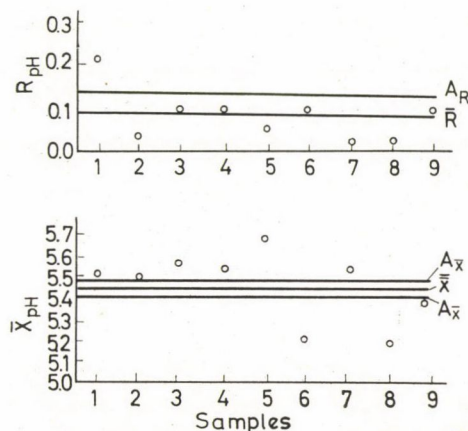


Fig. 1. Combined control card of average pH values and their range while controlling the production of canned food ("meat and vegetable thick soup")

bacteria, to use the maximum pH value permissible for the food in question (MAZOKHINA *et al.*, 1971).

Thermal resistance of *Cl. sporogenes* 25 spores has been determined at 108°C in the following canned foods:

— "Vegetable appetizer", cooked either without adding acid (pH = 4.45) or with acetic, citric and lactic acid additions (pH = 4.25, 4.25 and 4.00, respectively);

— "Borshch (beetroot and cabbage soup) from fresh cabbage without meat" (pH = 4.6);

— "Rassolnik (soup with pickled cucumbers) without meat" (pH = 4.8).

The same culture has been heated in the following canned foods at 121°C:

— "Borshch of fresh cabbage without meat" (pH = 4.6);

— "Rassolnik without meat" (pH = 4.8);

— "Shehi (cabbage soup) without meat" (pH = 4.9);

— "Rassolnik with meat" (pH = 5.1);

— "Borshch with meat" (pH = 5.46).

The results of heating are given in Fig. 2, showing that increasing the pH value increases the thermoresistance of *Cl. sporogenes*. For the calculation of the D value as a function of pH (in products with pH < 5.5) the following equations can be used:



$$D_{121^{\circ}} = 1.07 \text{ pH} - 4.0,$$

$$D_{108^{\circ}} = 1.39 \text{ pH} - 3.69.$$

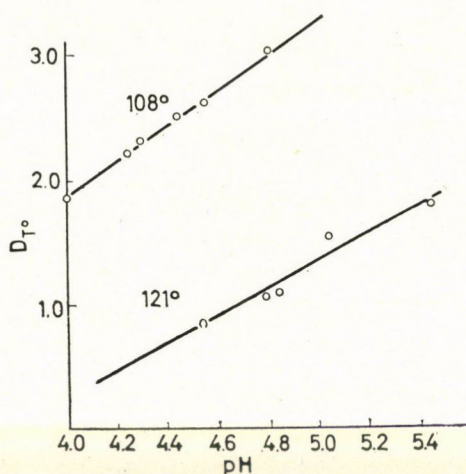


Fig. 2. Dependence of *Cl. sporogenes* 25 spore thermoresistance ( $D_T$  value) on the pH value of canned food

The results of experiments with *Cl. pasteurianum* 148 heated in phosphate buffer solution at  $95^{\circ}\text{C}$  are shown in Fig. 3.

These data reveal that *Cl. pasteurianum* 148 forms two types of spores, the thermolabile and the thermostable ones. Out of  $10^8$  spores,  $10^2$  are thermo-

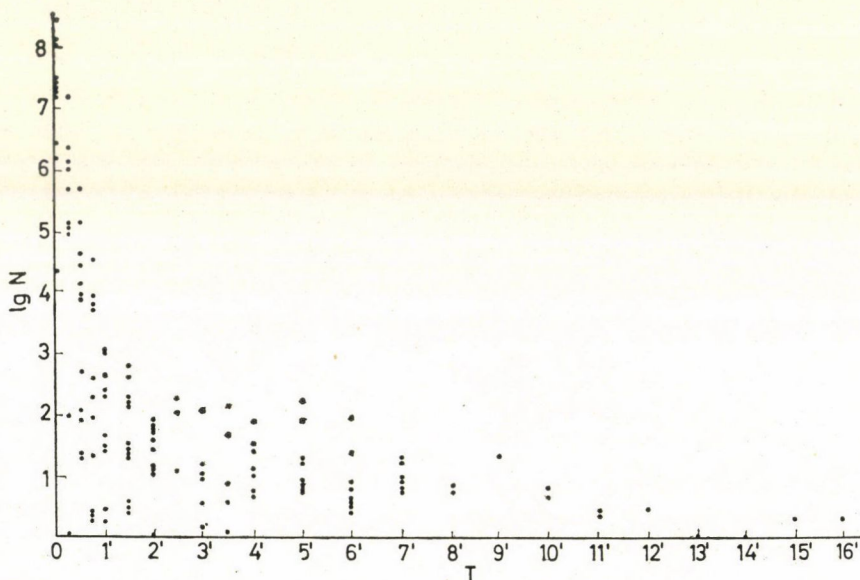


Fig. 3. *Cl. pasteurianum* 148 spore survival at  $95^{\circ}\text{C}$

stable cells. Counting spores of *Cl. pasteurianum* 148 surviving heat treatments at 95°C in citric or phosphate buffer solutions at various pH values (4.5, 5.5, 7.0) showed that  $D_{95^\circ}$  values for thermolabile spores were between 16 and 17 seconds and these did not depend on the active acidity of the medium. Use of the probit-method to determine the period necessary for the destruction of 50 per cent of thermostable spores, the  $LD_{50}$ , resulted in 70, 75 and 81 seconds in media of the pH values of 4.2, 5.5 and 7.0, respectively.

In addition, the pH effect on the thermal resistance of *Cl. botulinum* B-4 spores has been studied. *Cl. botulinum* B-4 spores have been heated at 103°C in different homogenized vegetable mixtures at pH values of 4.5 and 5.0. The obtained curves of spore survival in cabbage with tomato sauce are shown in Fig. 4. As can be seen, thermoresistance of spores at pH = 5.0 is given as a straight line in the semilogarithmic co-ordinate system and, accordingly, thermoresistance may be characterized by the  $D_{103^\circ}$  values of 30 and 34.5 seconds. The *Cl. botulinum* B-4 survival curve at a pH of 4.5 is not exponential.

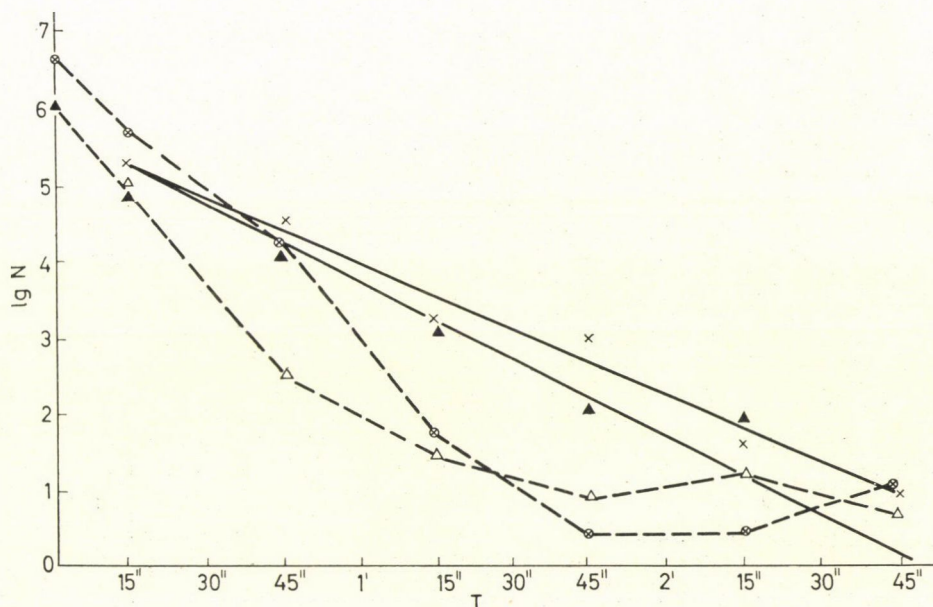


Fig. 4. *Cl. botulinum* B-4 spore survival curves in canned food of various pH values

	Cabbage : Tomato sauce	pH
— × —	2 : 1	5.0
— ⊗ —	2 : 1	4.5
— ▲ —	1 : 2	5.0
— △ —	1 : 2	4.5



### 3. Conclusions

The thermoresistance of *Bac. stearothermophilus* 80 and *Cl. sporogenes* 25 depends on the pH of the product. In the puree-like canned vegetable and vegetable and meat products the following D values can be calculated for *Bac. stearothermophilus*:

$$D_{121^{\circ}} = 1.7 \text{ pH} - 7.5.$$

In canned vegetable products ( $\text{pH} < 5.5$ ), for *Cl. sporogenes*:

$$D_{108^{\circ}} = 1.39 \text{ pH} - 3.69,$$

$$D_{121^{\circ}} = 1.07 \text{ pH} - 4.0.$$

The *Cl. pasteurianum* 148 population consists of thermolabile and thermostable spores. Heat resistance of thermolabile spores in the range of pH 4.5 to 7.0 is independent of the pH but that of the thermostable ones does depend on hydrogen ion concentration.

One of the hypotheses, explaining why no pH effect was found with thermolabile spores of *Cl. pasteurianum* 148 might be the assumption that the velocity of spore destruction exceeded that of ion-exchange, taking place between the spore and the medium (ALDERTON *et al.*, 1964).

Thermal resistance of thermostable spores of butyric acid bacteria might not be taken into consideration, when calculating the duration of heat treatment of tomato juice or tomato paste in cans. But the thermoresistance of thermostable spores should be taken into consideration for the aseptic packing in large tanks and barrels. In this case acidification of food might provide shorter sterilization periods.

Data on the pH effect on  $D_{121^{\circ}}$  for spoilage microorganisms can be used to calculate the length of sterilizing treatment. The pH value affects not only the thermoresistance of spores, but also their germination as well as culture development and can spoilage. That is why sterilizing periods for each kind of food, calculated according to the data obtained from equation for  $D_T$  dependence on pH, should be additionally checked and tested under commercial conditions on experimental batches of canned food.

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## THE EFFECT OF CYCLOHEXIMIDE ON THE RADIOSENSITIVITY OF MICROORGANISMS\*

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The influence of cycloheximide and gamma radiation was proved on the very radioresistant yeast strain *Saccharomyces cerevisiae* var. *ellipsoideus* using a semi-synthetic culture medium. A concentration of 5 ppm cycloheximide is necessary for total growth inhibition of this yeast strain within 72 hours. If this antibiotic treatment is followed by gamma radiation a synergistic effect occurs whereby, for instance a concentration of 1 ppm and an irradiation dose of 200 krad is sufficient to inhibit growth completely for this period of time.

For blastospores of *Pullularia pullulans* it could be found that a pre-treatment with cycloheximide causes a maximal radiation effect in which the shoulder in its radiation survival curve disappears. The same treatment after irradiation showed nearly no effect. Radiosensitive spores of microorganisms showing no or only a very weak shoulder in their radiation survival curves are nearly uninfluenced by cycloheximide in this concentration range.

By comparing alkaline sucrose gradient centrifugation patterns of DNA extracted from cells after such treatments it could be shown that this synergistic effect is caused by an interaction of the cycloheximide with the repair system of the yeast cell, probably with the repair enzymes.

One of the greatest difficulties in the use of radiopreservation for fruit juices is the fact that the irradiation doses necessary to kill microorganisms or to inhibit their growth is in the range of about 2 Mrad (FERNANDEZ-GONZALES *et al.*, 1966). If we consider on the other hand that the 500 krad range cannot generally be exceeded without inducing undesirable flavours, then it is clear that only combined sensitisation treatments would lead to success. We tried, therefore, to block DNA repair enzyme activities because in this case a maximum efficiency of a gamma radiation treatment would be attained.

Therefore a number of combination treatments of irradiation and chemicals were investigated. Most of them failed, however, because the biological activity was strongly affected by the irradiation treatment itself, as it was the case, for instance, with sorbic acid or pimaricin, but cycloheximide was found to be stable within the investigated dose range up to 500 krad. Cycloheximide is found in culture filtrates of streptomycin-producing strains of *Streptomyces griseus* and is very effective against yeasts and fungi in concentrations beginning with 0.2 ppm (WHIFFEN, 1948) but has almost no influence on bacteria

\* Presented at the IUFoST Symposium on Combination Treatments in Food Preservation, Budapest 18—22 September 1972.



<sup>u</sup>p to 1 000 ppm (see Table 1). Its structure was ascertained by KORNFIELD *et al.*, (1949) as 3-(2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl)-glutarimide:

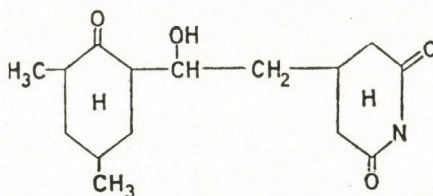


Table 1

*Cycloheximide (I)-concentrations completely inhibiting multiplication during 72 hours (Whiffen)*

Organism	Concentration ppm
<b>Yeasts:</b>	
<i>Saccharomyces carlsbergensis</i>	0.17
<i>Saccharomyces ellips. var. burgundy</i>	0.17
<i>Saccharomyces pastorianus</i>	0.17
<i>Rhodotorula glutinis</i>	0.31
<i>Saccharomyces cerevisiae</i>	0.4-10
<i>Torula utilis</i>	10
<i>Schizosaccharomyces pombe</i>	25
<i>Saccharomyces lactis</i>	>1 000
<b>Bacteria:</b>	
<i>Aerobacter aerogenes</i>	>1 000
<i>Bacillus subtilis</i>	>1 000
<i>Escherichia coli</i>	>1 000
<i>Salmonella schottmuelleri</i>	>1 000
<i>Staphylococcus aureus</i>	>1 000
<i>Streptococcus faecalis</i>	>1 000
<b>Phytopathogenic fungi:</b>	
<i>Cladosporium fulvum</i>	0.25
<i>Alternaria solani</i>	40
<i>Fusarium lycopersici</i>	100
<b>Pathogenic fungi:</b>	
<i>Cryptococcus neoformans</i>	0.24
<i>Phialophora verrucosa</i>	12.5
<i>Monosporium apiospermum</i>	25
<i>Blastomyces dermatitidis</i>	>1 000
<i>Candida albicans</i>	>1 000

Cycloheximide shows great differences also in its toxicity towards different strains of yeast. While *Saccharomyces fragilis* (Jorgensen) needs at least 900 ppm for slowing down its growth rate, this effect appears with *Saccharomyces pastorianus* (Hansen) already at concentrations lower than 1 ppm. This can be explained by the fact that *Saccharomyces fragilis* is not able to concentrate cycloheximide from extracellular solutions, while *Saccharomyces pastorianus* (Hansen) concentrates this substance within 30 minutes more than 10-fold when applied in concentrations of 0.1 or 1 µg/ml (SIEGEL & SISLER, 1964; WESCOTT & SISLER, 1964). The differences in behaviour of these two microorganisms are not attributed to different permeabilities of their cell walls, but to different sensitivity of their protein synthesis systems against cycloheximide.

## 1. Materials and methods

### 1.1. Test organisms

A radiation resistant strain of *Saccharomyces cerevisiae* var. *ellipsoideus* was used throughout these yeast experiments. *Byssoschlamys fulva* strain A-2614 and strain A-3849 were supplied by the Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, Illinois. *Pullularia pullulans* was of Danish origin. *Penicillium terrestre* (Jensen) was isolated from apple juice.

### 1.2. Nutrient mediums

Overnight cultures were prepared growing this yeast in a semi-synthetic nutrition medium containing:

	g
Potassium dihydrogen orthophosphate	1.0
Magnesium sulphate	0.5
Sodium chloride	0.1
Calcium chloride	0.1
Ammonium sulphate	5.0
Bacto yeast extract from Difco	7.0
Sucrose	50.0
dissolved in one litre of distilled water.	

### 1.3. Radiation treatment and yeast DNA separation

For investigating the DNA repair after gamma irradiation using a modified method of CABELA *et al.* (1971) this yeast was incubated in its logarithmic growth phase for 2 hours at 30°C in the semi-synthetic nutrition medium containing 1.0 ppm cycloheximide. After two washings with 0.14 M potassium phosphate buffer of pH 6.0 samples with 0.1 g (moist weight) were suspended



in 0.04 *M* potassium phosphate buffer pH 7.3 and irradiated at 4°C within 40 minutes with a total irradiation dose of 300 krad. Samples were deep-frozen immediately after irradiation treatment and after incubation times of 10, 20, 30 and 40 minutes, respectively, at 37°C in the darkness. After homogenizing the cells DNA was extracted with NaCl and phenol. For this purpose 0.5 ml of a solution containing 5 *N* NaCl and 1% Na desoxycholate, 4 g glass powder (0.5 mm in diameter) and 1.0 ml phenol were added to each deep-frozen sample. Homogenization was carried out at 4°C using an apparatus of Merckenschläger (2 minutes at 4 000 vibrations/min). After centrifugation the supernatant (about 0.8 ml) was extracted again with phenol and then 4 times with diethyl ether. After removing traces of this ether from the aqueous layer 0.4 ml samples were brought onto a linear sucrose gradient containing 5–20% sucrose in 0.9 *M* NaCl + 0.001 *M* EDTA + 0.1 *M* NaOH (corresponding to a density range from 1.074 to 1.113) and centrifuged during 15 hours at 35 000 rpm (179 000  $\times g$ ) in a Beckman L2-65b ultracentrifuge using the swingout rotor SW 40 Ti at 5°C. For evaluating the DNA fractionation the equilibrated solutions were carefully pumped from the centrifuge tubes through a glass capillary system into microcuvettes for continuous flow of a Zeiss PMQ II spectrophotometer using a peristaltic pump, and optical density was measured at 260 nm against water.

Spores were irradiated (460 krad/hour) at 0°C in 0.1 ml citrate buffer pH 4.6 with or without cycloheximide pre-treatment (incubation at 27°C for 1 hour).

## 2. Results

### 2.1. Influence of cycloheximide and radiation on the multiplication of *Saccharomyces*

Table 2 shows the influence of the concentration of cycloheximide on the multiplication of this yeast strain as found by CABELA *et al.* (1967). Growth is only slightly inhibited by a concentration of 0.1 ppm which corresponds to  $3.5 \times 10^{-7}$  mol/l. Even at a concentration of 0.75 ppm growth may be still observed. At 1.0 ppm the viable cell count declined over the initial growth period, while at 5 ppm and above no living cells could be detected after an incubation of 72 hours.

The influence of gamma irradiation alone on the surviving and growth of *Saccharomyces cerevisiae* can be seen in Fig. 1. Even at a dose of 200 krad there was only a slight inhibition of growth.

In Fig. 2 the effect of combined cycloheximide and irradiation treatment on the growth of this yeast may be seen as it was found by CABELA *et al.* (1967). The decrease in cell number is more than two orders of magnitude

Table 2

*Influence of cycloheximide concentrations on the multiplication of Sacch. cerevisiae var. ellipsoideus cultured in a semisynthetic medium at 27°C*

Cycloheximide (ppm)	Cell counts*			
	4 hours	24 hours	48 hours	72 hours
0	$1.7 \times 10^4$	$1.6 \times 10^7$	$3.3 \times 10^8$	$3.8 \times 10^8$
0.1	$1.1 \times 10^4$	$3.8 \times 10^6$	$4.8 \times 10^7$	$8.0 \times 10^7$
0.2	$1.0 \times 10^4$	$5.5 \times 10^6$	$1.7 \times 10^7$	$1.9 \times 10^7$
0.3	$9.0 \times 10^3$	$6.0 \times 10^5$	$7.5 \times 10^6$	$5.0 \times 10^6$
0.5	$7.0 \times 10^3$	$3.3 \times 10^4$	$9.5 \times 10^5$	$5.5 \times 10^5$
0.75	$6.5 \times 10^3$	$1.6 \times 10^4$	$5.5 \times 10^5$	$9.0 \times 10^4$
1	$5.0 \times 10^3$	$3.8 \times 10^3$	$2.5 \times 10^3$	$6.5 \times 10^3$
2	$4.9 \times 10^3$	$2.1 \times 10^3$	$1.9 \times 10^3$	$3.0 \times 10$
3	$4.3 \times 10^3$	$1.6 \times 10^3$	$1.4 \times 10^3$	6.5
5	$4.2 \times 10^3$	$8.5 \times 10^2$	$1.8 \times 10^2$	+
7.5	$3.8 \times 10^3$	$4.1 \times 10^2$	9.0	+
10	$3.3 \times 10^2$	$2.5 \times 10^2$	4.4	+

\* Initial cell count at zero time was approx.  $5 \times 10^3$  cells/ml

+ No survivals detected

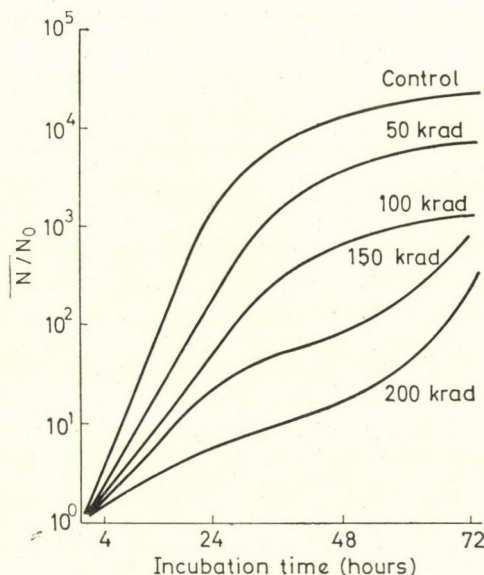


Fig. 1. Multiplication of *Sacch. cerevisiae* var. *ellipsoideus* in a semi-synthetic culture medium after different doses of gamma radiation

3 days after irradiation with 200 krad at a cycloheximide concentration of 0.75 ppm; the non irradiated sample has by then increased its cell number by



a factor of 20. Even with a dose of 50 krad a marked decrease in cell number occurs within 20 hours after irradiation. A similar decrease is obtained with 0.5 ppm at 100 krad. When the yeasts are incubated with 1 ppm cycloheximide and irradiated with 200 krad, no survivors are found after 3 days.

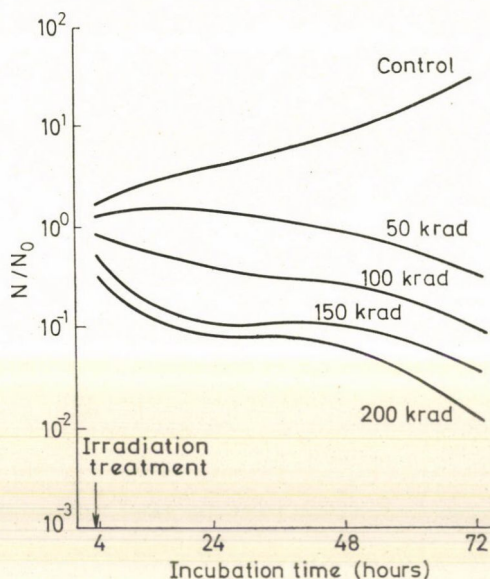


Fig. 2. Multiplication of *Sacch. cerevisiae* var. *ellipsoideus* in a semisynthetic culture medium with 0.75 ppm of cycloheximide

The influence of different concentrations of cycloheximide on the  $LD_{90}$  values of *Saccharomyces cerevisiae* immediately after irradiation treatment is shown in Table 3. The corresponding  $LD_{90}$  value for 0.5 ppm cycloheximide

Table 3

*The influence of different concentrations of cycloheximide in the culture medium on the  $LD_{90}$  values of *Sacch. cerevisiae* var. *ellipsoideus**

Cycloheximide (ppm)	$LD_{90}$ (krad)
0	420
1	201
2	157
3	132
5	100
7.5	88
10	80

would be 265 krad. With increasing concentrations of this substance a marked decrease of these  $LD_{90}$  values may be observed which approach 80 krad with 10 ppm cycloheximide. This fact is very interesting because this yeast strain shows such an  $LD_{90}$  value also when culture media are used for irradiation in which no repair can take place (FÁBRI *et al.*, 1972) or when only the slope of that part of the survival curve is considered which follows the shoulder.

## 2.2. Influence of cycloheximide on the radiosensitivity of different spores

For blastospores of *Pullularia pullulans*, an extremely radioresistant microorganism ( $LD_{90} = 540$  krad) showing a large shoulder in its radiation survival curve and indicating by this a marked capacity of repair enzymes, it could be found by ZECHMEISTER *et al.* (1972), that a 1 hour pre-treatment with cycloheximide causes a maximal radiation effect in which this shoulder disappears (Fig. 3). The same treatment applied after irradiation shows nearly

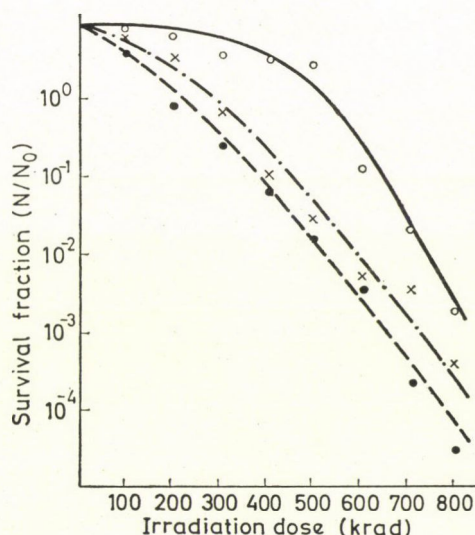


Fig. 3. Influence of cycloheximide on the radiation survival of blastospores of *Pullularia pullulans*. ○—○ Irradiation without cycloheximide treatment; ×—·—× irradiation and pre-treatment (1 hour incubation in 20 µg/ml cycloheximide); ●—— irradiation and pre-treatment (1 hour incubation in 40 µg/ml cycloheximide)

no effect. The survival rate of these spores, even after a 3-day incubation period with 20 µg/ml cycloheximide, comes to about 70%.

Radiosensitive spores of microorganisms showing no or only a very weak shoulder in their radiation survival curves are nearly uninfluenced by cycloheximide in this concentration range (Table 4).



Table 4

*Influence of cycloheximide pre-treatment  
on the radiation sensitivity of spores of different organisms*

	LD <sub>50</sub> (in krad)		
	γ-irradiation without cycloheximide	1 hour pre-incubation at 27°C with cycloheximide	
		20 µg/ml	40 µg/ml
Blastospores of <i>Pullularia pullulans</i>	540	300	235
Conidiospores of <i>Penicillium terrestre</i>	50	47	—
Conidiospores of <i>Byssoschlamys fulva</i> strain 2614	36	25	—
Ascospores of <i>Byssoschlamys fulva</i> strain 3849	53	50	—

### 2.3. Investigation of DNA repair of *Saccharomyces*

We tried, therefore, to ascertain if this synergistic effect of a combined cycloheximide — irradiation treatment was caused by an interaction with the repair system of these microorganisms. Fig. 4 shows alkaline sucrose gradient centrifugation patterns of DNA extracted from yeast cells after such treatments. It can be seen that the peak of single strand DNA of this yeast normally accumulates at a sucrose density of 1.094. After an irradiation treatment with 300 krad at 4°C its density shifts, due to the formation of various structural defects in DNA, to 1.086, if DNA is isolated immediately after the irradiation treatment. This change in the sedimentation profile is mainly due to single strand breaks of this macromolecule; short DNA fragments have a lower sedimentation velocity in this system. If enzymes are allowed to repair or bypass any individual but potentially lethal structural defect of DNA, as was done for instance by raising the temperature of the cells after the irradiation treatment for some minutes to 37°C, the density shift of this DNA slowly decreases with increasing incubation time, reaching its original value after a repair incubation time of somewhat above 30 minutes. This kind of repair of radiation-damaged DNA normally should be finished, therefore, after such a period of time.

If these yeast cells are incubated before the irradiation treatment for 2 hours in a culture medium containing 1 ppm cycloheximide which allows recovery only to a small extent, the repair-results, as could be seen, are quite different. Only a small correction of this radiation induced DNA shift could be reached, even after the double incubation time of 60 minutes, whereas the slight shift after 40 minutes might be contributed mainly to freshly synthesized DNA.

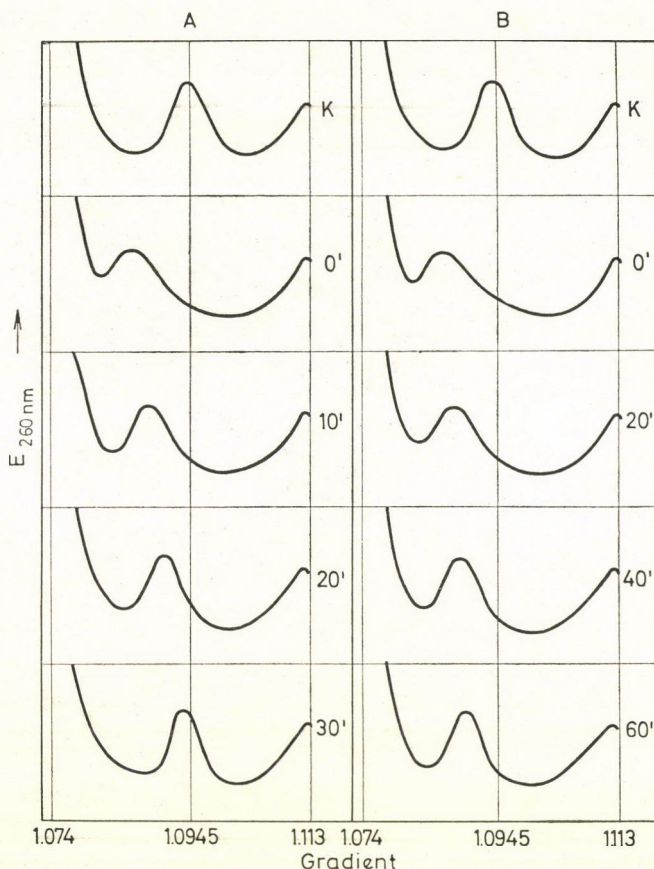


Fig. 4. Sucrose density gradient ultracentrifugation pattern of nucleic acids from *Saccharomyces cerevisiae* var. *ellipsoideus* after a gamma irradiation dose of 300 krad at 4°C (A) without and (B) with pre-treatment (1 ppm cycloheximide for 2 hours). K unirradiated control, 10'–60' time interval at 37°C after irradiation treatment to allow action of repair enzymes

### 3. Conclusions

These results can be interpreted by the fact that repair of the radiation damaged DNA molecule is blocked by cycloheximide in this concentration range. The other possibility, namely that cycloheximide combines with DNA in such a way as to render degradation more rapid and effective, is rather improbable, because there is no evidence that this compound is capable of binding to DNA (LEE-BENNETT *et al.*, 1964). It inhibits either existing DNA repair systems or the synthesis of any repair enzyme that would be induced by the action of radiation.

By the results obtained with spores of different microorganisms and by the results of KERRIDGE (1958), who found in yeast cells that protein synthesis



was inhibited by cycloheximide, it appears to be most probable that synthesis of repair enzymes is blocked by cycloheximide. A short pre-incubation of cells will then lead to a deficiency of these enzymes and thus to a marked increase of their radiosensitivity.

The observed synergistic effect in a combined cycloheximide + irradiation treatment can be attributed in any way to the inhibition of enzymatic reactivation processes which otherwise would serve to repair or bypass some fractions of the macromolecular damage.

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## THE EFFECT OF SOME GROWTH REGULATORS ON ENZYME SYSTEMS IN IRRADIATED BARLEY GRAIN USING DISINFESTATION DOSES\*

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Disinfestation doses of 20 to 100 krad may cause changes in the biological systems of barley grain and, therefore, may influence undesirably the technological quality of malted grain. The effect of some growth regulators on irradiated grain has been investigated. The experiments have been carried out on brewery barley var. Visa Breuns. Following growth-regulators were used: gibberellic acid (Polish preparation "Gibrescol"), kinetin (6-furfurylo-aminopurin), CCC (2-chloroethyl trimethyl ammonium chloride), and betaine hydrochloride. By treating the irradiated barley with solutions of growth regulators it was possible to diminish the loss of enzyme activity. A "regenerating" effect of growth substances, mainly gibberellic acid and betain hydrochloride in  $10^{-4}$  M solutions, was observed. Amylolytic activity decreased immediately after irradiation but in samples treated with growth regulators it was higher than in those without regulators. The results may have a practical importance since gibberellic acid has just been introduced into the brewery industry.

Contemporary biochemistry is becoming more and more connected with the problems of food technology. The biochemical studies concern not only the chemical composition of food products but also metabolic processes. In order to follow the biochemical changes different reagents are used, *e.g.* growth regulators, antibiotics etc. Physicochemical methods based on the application, *e.g.* of low and high frequency vibrations and of electromagnetic radiation including gamma radiation serve equally to this purpose. The joint application of ionizing radiation and growth substances produce certain effects. Preliminary studies on the influence of growth regulator treatment combined with irradiation were initiated in our laboratory in 1968.

The doses of ionizing radiation which are efficient in disinfestation have the ability to decrease the germination of grain. Thereby they impair the enzymatic processes in grain used for malting. Many literary data are available on the influence of irradiation and growth regulators, especially gibberellic acid ( $\text{GA}_3$ ), as applied separately to barley malt. CORNWELL (1966) observed that at a dose of 8 krad the germination was disturbed and at 50 krad greatly inhibited; as a general rule, the grains which have been exposed to ionizing radiation in a linear accelerator in the dose range of 10 to 100 krad do not germinate.

\* Presented at the IUFOST Symposium on Combination Treatments in Food Preservation, Budapest, 18—22 September 1972.



TIPPLES and NORRIS (1963) studied the effect of different doses on barley which had been stored for some time after irradiation, and noticed a relatively weak effect of 50 krad on the germination. It is also possible to make use of irradiation in order to modify the biochemical processes in grain (BRUDZYNSKI, 1963; FARKAS *et al.*, 1963). KEMPE and GRAIKOSKI (1964) assume that irradiation may be a very efficient way of radurization of barley malt. FRANK *et al.* (1966) have found some changes in the protein fractions of grain and a decrease in the activity of amylolytic and proteolytic enzymes. Enzymatic systems of grain irradiated with 50 to 100 krad were also studied by BANCHER *et al.* (1970), they have found a decrease in enzyme activity.

For many years gibberellic acid has been used in the brewing industry of different countries to shorten malting time. PALEG (1960), YOMO (1960), VARNER and RAM CHANDRA (1964) showed that during the germination of grain, the embryo secretes to the aleurone layer gibberellic acid which plays the role of a stimulating agent in  $\alpha$ -amylase accumulation. VARNER *et al.* (1965) reports on the production of  $\alpha$ -amylase and other enzymes in the grain endosperm under the influence of GA. FAUST and MASSEY (1966), MACLEOD *et al.* (1966), VIGIL and RUDDAT (1970) have also found an increased production and secretion of enzymes, especially of hydrolases in the aleurone tissue treated with GA. LÜTTGE *et al.* (1968) suggested that membranes and the transport mechanism through membranes of young plants were affected by GA. The magnitude of this effect depends on the time of GA action. JACOBSEN *et al.* (1970) observed that in the presence of GA isolated layers of barley cells produced and secreted 4 kinds of  $\alpha$ -amylases into the medium. In spite of many communications, the mechanism of gibberellic acid biosynthesis in the natural, biological systems has not been elucidated so far. Neither is the mechanism of GA action known in detail. Presumably, gibberellic acid affects the production of specific m-RNA, connected with the synthesis of enzymes which in turn causes changes in the metabolism of plants (MACIEJEWSKA-POTAPCZYK, 1967; KOPCEWICZ, 1970).

SIDERIS *et al.* (1971) irradiated aqueous solutions of gibberellic acid with doses inducing  $\alpha$ -amylase activity in barley detached from its embryos. They found a linear relationship between radiation doses and inactivation values of GA. The products of the radiolysis of water, mainly OH-radicals are responsible for the inactivation of GA.

The attempts of treating the grain with growth regulators as retardants showed — as stated by KNYPL (1970) — no inhibition of  $\alpha$ -amylase synthesis specifically induced by GA in the aleurone layer. From this fact it follows that the above mentioned compounds do not block the physiological function of gibberellic acid. GALSKEY and LIPPINCOTT (1970) made attempts to induce  $\alpha$ -amylase activity by steeping barley in solutions of some basic amino acids.

The review of communications given in the introduction and concerning

the effects of radiation on barley used for malting and previously treated with gibberellic acid is only a short approach to the problem as treated by different authors.

## 1. Materials and methods

The 1967 crops of the barley var. Visa Breuns used in our studies was tested to have the following characteristics:

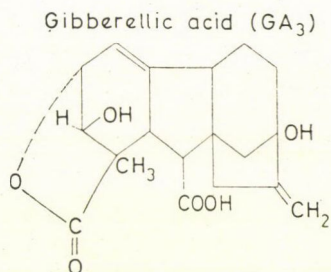
humidity — 14.3%, ash — 1.75%, protein ( $N \times 6.25$ ) 11.18%, germination ability — 99.3%, weight of 1 000 seeds — 39.7 g.

### 1.1. Growth regulators

The following growth regulators were used:

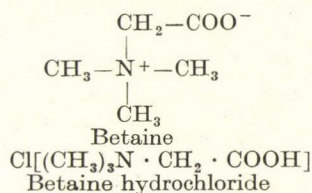
— Gibberellic acid ( $GA_3$ ), Polish preparation "Gibrescol" from PoLFA, Kutno,

Formula 1



— Betaine hydrochloride (BA) — Polish preparation LZS — Lublin

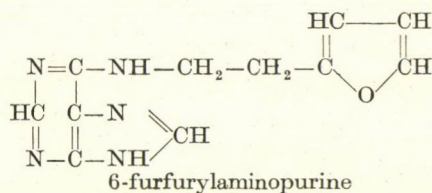
Formula 2



— Kinetin (KA) 6-furfurylaminopurine from Schuchard, GDR

Formula 3

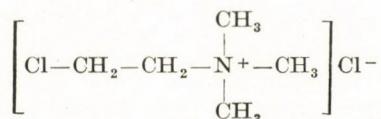
Kinetin





— 2-chloroethyl trimethyl ammonium chloride (CCC), GDR preparation.

Formula 4



2-chloroethyl trimethyl ammonium chloride

### 1.2. Procedure of irradiation

Barley in 100-g samples was irradiated in polyethylene bags, in air at room temperature at the dose rate of 120 krad/hour in the  $^{60}\text{Co}$  radiation source of the Institute of Applied Radiation Chemistry in Łódź (the total activity of the sources was 20 kCi).

Extracts of grain were prepared in buffered aqueous solutions at  $\text{pH} = 7.2$  and in the solutions of growth regulators of the final concentration of  $10^{-4} M$ . The concentration of GA in such extracts was  $10^{-5}$  and  $10^{-6} M$ , respectively.

The amylolytic activity was determined by measuring the reducing sugars produced by the action of amylases on a 1% starch solution. Amylolytic activity was expressed in relative units as per cent of total activity of extracts prepared in buffered aqueous solutions from not irradiated control grain samples.

The reducing sugars were determined colorimetrically using 3,5-dinitro-salicylic acid (DNS) as reagent (DALQUIST, 1961). The colour intensity was measured at 530 nm.

## 2. Results

Figs. 1 and 2 shows the amylolytic activity of the grain at different times after irradiation. There is a minimum of activity after 12 hours, growth to a maximum at about 48 hours, a second minimum and after 10 days nearly the same value as at the starting point. This suggests a spontaneous enzyme repairing ability in the natural environment. The smallest decrease in amylolytic activity or even some stimulating effect can be observed with the doses of 40 to 60 krad. This could be in agreement with the observations of COEHLO (1969) as to the existence of specific dose ranges which stimulate the activity of some enzymes.

A few growth regulating compounds as gibberellic acid, betaine hydrochloride, kinetin and CCC have been applied to check their ability to "regenerate" the amylolytic activity damaged by the action of ionizing radiation. The results presented in Table 1 show a positive action of gibberellic acid and betaine in the range of all doses applied, particularly from 40 to 60 krad. Kinetin shows rather a small positive effect in the range of 20 to 40 krads and unexpectedly CCC had a better effect than kinetin.

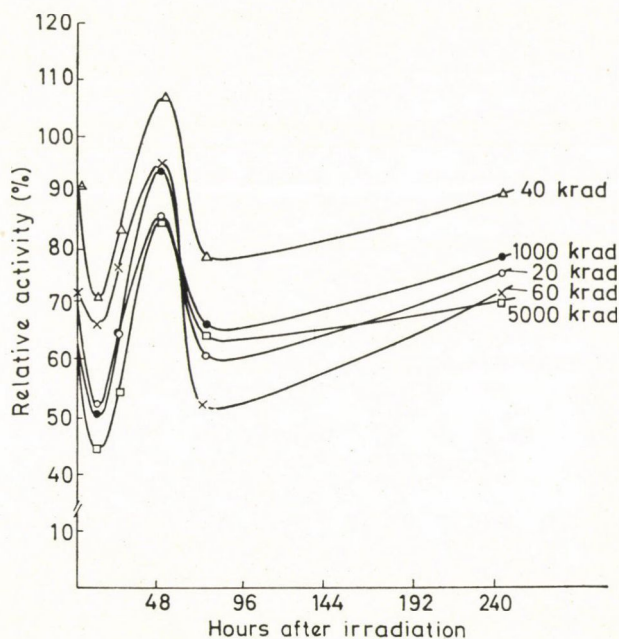


Fig. 1. Amylolytic activity of barley extracts at different times after irradiation

In our studies special attention was paid to the influence of GA (Gibberescol) and to betaine hydrochloride (BA). These are manufactured in Poland and especially BA is easily available and relatively inexpensive. Their action on amylolytic activity has been investigated at different periods after irradiation and for various gibberellic acid concentrations ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  M) (Figs 3, 4, 5).

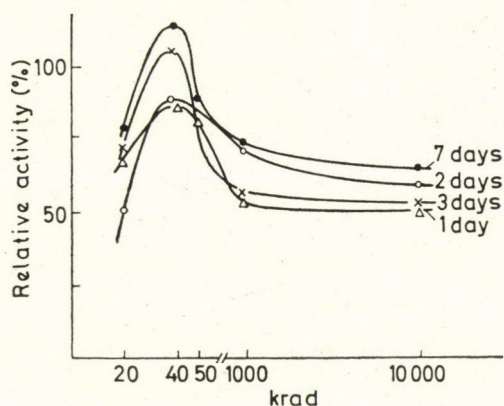


Fig. 2. Amylolytic activity of barley extracts, after irradiation of grain with various doses



Table 1

*Effect of different growth regulators on the extracts of barley irradiated with various doses*

Dose (krad)	Relative amylolytic activity, %*				
	H <sub>2</sub> O	GA <sub>3</sub>	BA	KA	CCC
0	100	258.0	99.5	108.0	90.4
20	63.6	140.0	163.5	66.9	61.0
40	98.7	227.0	198.0	99.6	103.1
60	81.8	463.0	296.0	60.6	78.5
100	70.6	360.3	290.8	60.0	72.0
1 000	64.5	258.0	128.5	48.2	68.6
10 000	58.7	195.2	68.5	42.2	65.7

GA<sub>3</sub> = gibberellic acid

BA = betaine hydrochloride

KA = kinetin

CCC = 2-chloroethyl trimethyl ammonium chloride

\* Amylolytic activity was related to the control sample not irradiated.

It was observed that the most efficient concentration of GA for lower doses of irradiation, *e.g.* 20 krad was  $10^{-5}$  *M*, for the higher ones, 60 krad,  $10^{-4}$  *M*. The most efficient concentration of GA increased with higher doses of irradiation.

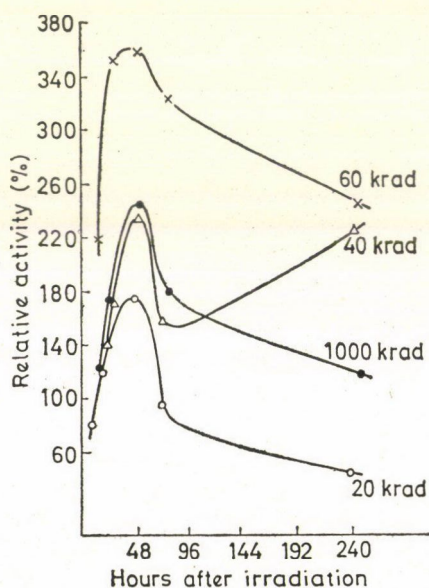


Fig. 3. Amylolytic activity of barley extracts in the presence of  $10^{-4}$  *M* gibberellic acid

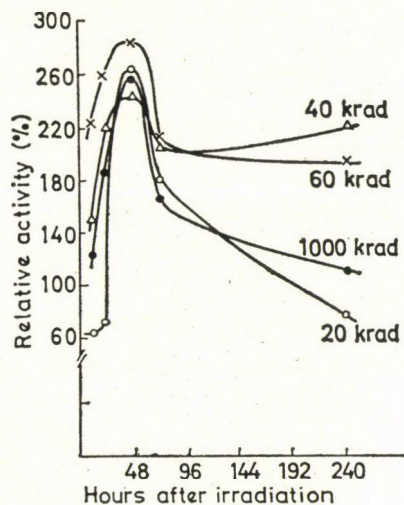


Fig. 4. Amylolytic activity of barley extracts in the presence of  $10^{-5}$  M gibberellic acid

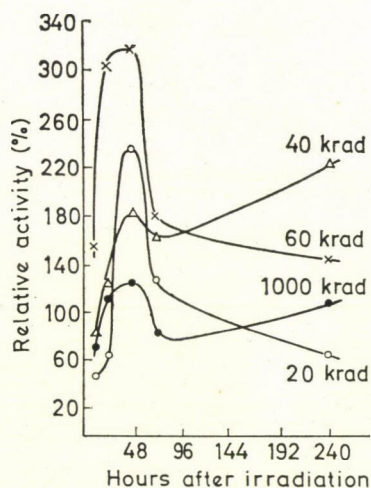


Fig. 5. Amylolytic activity of barley extracts in the presence of  $10^{-6}$  M gibberellic acid

The action of betaine hydrochloride in the concentration of  $10^{-4}$  M was nearly as good as that of gibberellic acid in the elimination of effects caused by ionizing radiation, especially in the dose range from 40 to 60 krad (Fig. 6).

### 3. Conclusions

The results of our investigations presented in this paper contain a series of observations without the explanation of the reaction mechanism. The growth substances may be used for regeneration of amylolytic activity in the grain damaged by irradiation. The results point to the necessity of further



studies concerning the processes which occur in the irradiated grain. The influence of radiation and gibberellic acid on the changes of amylolytic activity in the grain should be associated with the processes in the permeability of cellular membranes in grain tissues and also with the disturbances of the action of endogenous gibberellins which are responsible for the induction of  $\alpha$ -amylase synthesis (LÜTTGE *et al.*, 1968; KUZIN & TAGI-ZADE, 1971). The addition of growth regulators during the process of grain steeping may counterbalance possible negative effects of ionizing radiation.

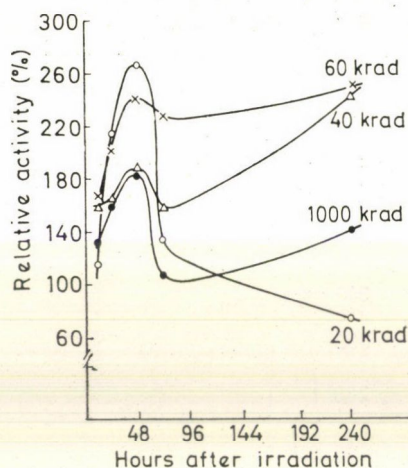


Fig. 6. Amylolytic activity of barley extracts in the presence of  $10^{-4}$  M betaine hydrochloride

Retardants, as for instance CCC used in our studies do not inhibit the synthesis of  $\alpha$ -amylase specifically induced by endogenous gibberellins (PALEG *et al.*, 1965).

The very interesting effects caused by the action of betaine require further studies. Betaines, quarternary ammonium bases, belong to the group of very important compounds. Betaine hydrochloride is the by-product of beet sugar manufacture and molasses distillation. The use of this compound may be of great interest both for the malting technology and for other branches of the food industry where irradiated grain is applied and negative radiation post-effects should be eliminated. So far there are no publications concerning this problem. More detailed studies, as required, are in progress.

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## STUDIES INTO THE O-DIPHENOL OXIDASE ACTIVITY OF POTATOES

### PART I. — DEVELOPMENT OF AN ENZYME ACTIVITY ASSAY METHOD

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1. A simple method suitable for routine analysis was developed to measure the activity of potato o-diphenol oxidase (polyphenol oxidase). The method is based on the spectrophotometric determination of optical density of the coloured compounds formed from the substrate by enzyme action.

2. Pyrogallol was used as substrate.

3. The optimal conditions of measurement were established as follows: 3.2% w/v substrate concentration and 2% w/v potato content in the reaction mixture, pH 6.2, temperature 30°C, incubation from 0 to 60 min under shaking, sampling every 10 or 20 min, optical densities taken at 400 nm against substrate solutions buffered to pH 6.2 and incubated for identical periods.

4. Under the conditions of the experiments reaction rate was found to be constant in the interval of 0—60 min (Fig. 5).

5. In the interval of 0—60 min of reaction time the optical densities of the reaction mixtures of 0.5—2.0% w/v potato content are in linear relationship with potato concentration (Fig. 6).

6. In the range investigated, reaction rate increases with increasing pH, up to the value of 7.3, thus measurement at higher pH values is more sensitive (Fig. 8). It is, however, more suitable to carry out activity determinations at pH 6.2, since this value corresponds to the original pH of the potato.

7. At the end of the incubation period enzyme inactivation in the reaction mixture is unnecessary; the optical density of the mixture, when kept in the refrigerator at +5°C, remains constant during 3 hours (Table 2).

8. The degree of enzyme activity is indicated by the slope of the regression line, as calculated from the optical density values measured after different incubation periods, *i.e.*, activity is expressed by the rate of change in optical density. Unit enzyme activity is defined as producing a change of  $1 \cdot 10^{-4}$  optical density modulus per minute.

9. The variation coefficient of the method is about 10—12%, 2/3 of this value being due to the deviations in comminution and subsequent pipetting of the potato suspension.

10. The polyphenol oxidase content of a spring sample of the potato variety Gül baba was found to be  $710 \text{ U} \cdot \text{g}^{-1}$ , as assessed by the method developed.

The extensive literature dealing with polyphenol oxidase (1.10.3.1. o-diphenol: oxygen oxidoreductase, hereafter ODO = o-diphenol oxidase) describes but a relatively small number of activity assay methods. The great majority of these methods is based upon the following principles:

a) Manometric measurement of oxygen uptake due to enzyme action (DRAUDT & HUANG, 1966; ENKELMANN, 1969; HAREL *et al.*, 1964; HYODO & URITANI, 1965; JONES *et al.*, 1965; MONDY *et al.*, 1966), polarographic (HAREL *et al.*, 1964; HEIMANN & ANDLER, 1962) or potentiometric (HYODO & URITANI, 1965) assay of the same;



b) Measurement of the colour intensity of the compounds formed from the substrate by enzyme action;

c) Indirect measurement of enzyme action by assessing the decrease of the amount of reducing substance added to the enzyme — substrate system.

The methods belonging to group *a*) are generally applied to determine the activity of enzyme preparations. In this case the rate of substrate oxidation as catalysed by the enzyme, *i.e.* enzyme activity, may be satisfactorily characterized by the rate of oxygen uptake. These methods are, however, less suitable to assess enzyme activity in tissues where oxidation processes independent from the enzyme reaction investigated, such as endogenous respiration, may lead to erroneously high activity values.

The colorimetric methods belonging to group *b*) are more widely used to measure enzyme activity in tissues. The substrate applied is, in most cases, some phenolic substance occurring in notable concentration in the tissue investigated, *e.g.* chlorogenic or caffeic acid (VOIGT & NOSKE, 1966), but simpler o-di- or -triphenols, such as pyrocatechol (VOIGT & NOSKE, 1966; BIEDERMANN, 1956), pyrogallol and, incidentally, monophenols, *e.g.* p-cresol, are applied as well.

The advantage of the colorimetric methods consists in that they are simple and not too time-consuming procedures and thus lend themselves to routine analysis. Their common drawback consists in the fact that the colour reaction, upon which the measurement is based, is brought about by a series of primary, secondary, etc. reaction products of different molecular weight and structure and thus the measurement of optical density gives but indirect information as to the rate of the enzyme reaction. Furthermore, at low pH values colour formation is not proportional to oxygen uptake (ENKELMANN, 1969).

Of the methods within group *c*) the best known is the one in which ascorbic acid is added to the o-di-phenol — polyphenol oxidase system and enzyme activity is characterized by the oxidation rate of the former. Ascorbic acid concentration is determined spectrophotometrically on the basis of light absorption at 265 nm (PATIL & ZUCKER, 1965). Although this method is simple, quick and sensitive, in principle the objection can be raised that ascorbic acid is — at least under certain conditions — an inhibitor of the enzyme (VOIGT & NOSKE, 1966). Consequently, *e.g.*, with apples, no relationship was found between the value of ODO-activity thus established and browning tendency. In a variant of this method potassium ferrocyanide was, therefore, applied instead of ascorbic acid. The oxidation of potassium ferrocyanide was followed by measuring the increase in optical density at 420 nm. The sensitivity of this variant is, however, but half as high as that of the ascorbic acid method (SUSSMAN, 1961).

For the purposes of the present study — the determination of ODO-enzyme activity in fruit and vegetable tissues — a simple and quick, well



reproducible method suitable for routine analysis had to be chosen, therefore it was decided to use the method based on the measurement of the colour intensity of the reaction products. Easily available and inexpensive o-di- or -triphenols, such as pyrocatechol and pyrogallol were tried as substrates, from which the latter was found more suitable due to its higher light absorption maxima. The method of activity measurement was developed for potato ODO.

## 1. Materials and methods

### 1.1. Materials

*1.1.1. Potatoes.* Batches of the potato variety "Gül baba" as purchased from primary producers were used throughout the experiments. To ensure reproducibility of the measurements only non-sprouted, intact tubers were taken from the batches.

*1.1.2. Substrate.* Daily prepared 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0% w/v buffered solutions of analytical grade pyrogallol manufactured by Carlo Erba (Milan) were used as substrate.

*1.1.3. Buffers.* 0.1 M, pH 4.0 citric acid-sodium citrate buffer (13.1 ml of 0.1 M citric acid + 6.9 ml of 0.1 M trisodium citrate, DAWSON *et al.*, 1959) was used to prepare the potato suspension.

Pyrogallol was dissolved — depending on the pH desired — in one of the following buffers (DAWSON *et al.*, 1959):

0.1 M, pH 7.0 phosphate buffer (61.0 ml of 0.2 M  $\text{Na}_2\text{HPO}_4$  + 39.0 ml of 0.2 M  $\text{NaH}_2\text{PO}_4$ , made up with water to 200 ml);

0.1 M, pH 8.0 phosphate buffer (94.7 ml of 0.2 M  $\text{Na}_2\text{HPO}_4$  + 5.3 ml of 0.2 M  $\text{NaH}_2\text{PO}_4$ , made up with water to 200 ml);

0.1 M, pH 9.0 glycine-buffer (50 ml of 0.2 M glycine + 8.8 ml of 0.2 N NaOH, made up with water to 100 ml).

All the solutions were prepared with boiled, twice distilled water.

### 1.2. Methods

*1.2.1. Preparation of the reaction mixture.* Three potato tubers each were peeled and cut into small cubes with a stainless steel knife. The diced potato was kept under water until further utilization (if this took place after more than 1 hour, in the refrigerator), to prevent browning.

10 g portions of the comminuted potato dried on the surface with filter paper were homogenized for 3 minutes with 90 ml pH 4.0 citrate buffer each in an "Atomix" homogenizer equipped with knives. Before pipetting, the potato suspension was heated to 30°C and mixed by shaking.

8 ml of the buffered substrate solution preheated to 30°C were mixed with 2 ml of enzyme suspension. Thus potato concentration in the reaction



mixture was always 2% w/v, with the exception of the series of experiments carried out in order to establish the relationship between enzyme concentration and activity (para. 2.4.).

In this latter series of experiments 0.5, 1.0, 1.25, 1.5, 1.75 and 2.0 ml, respectively, of the suspension containing 10 g potatoes and 90 ml pH 4.0 citrate buffer, were made up to 2.0 ml with pH 4.0 citrate buffer and mixed with 8 ml of substrate solution. Consequently, these mixtures contained 0.5, 1.0, 1.25, 1.5, 1.75 and 2.0% w/v of potatoes, respectively.

Varying the concentration of the pyrogallol solution between 1.0 and 4.0% w/v yielded substrate concentrations in the range of 0.8–3.2% w/v in the reaction mixture.

Depending on the pH value of the substrate the pH of the reaction mixture varied as follows:

pH of the substrate	pH of the reaction mixture
7.0	6.2
8.0	6.7
9.0	7.3

On applying the pH 9.0 glycine buffer, the pH 4.0 citrate buffer was used in a dilution of 1 : 1.

pH was checked in each series of measurements in a Metrohm E 166 pH-meter.

The mixture of 8 ml substrate solution and 2 ml pH 4.0 buffer was used as blank. The identity of the pH values of blank and reaction mixture was checked in every case.

*1.2.2. Determination of enzyme activity.* The reaction mixtures containing the enzyme, as well as the blanks were incubated in open, wide-necked Erlenmeyer flasks at 30°C for 0 to 4 hours under shaking (0.036 mmole O<sub>2</sub>/min) in the water bath "Vibroterm" manufactured by Labor-MIM. In some of the series of measurements samples were taken, i.e. one flask each was removed every 30, in others every 5 or 10 minutes. The contents of the flasks were filtered through a folded filter and optical density of the filtrate was read in a MOM 203 spectrophotometer against the blank incubated for the same period.

Three reaction mixtures prepared from the same potato suspension were incubated for the same period, in parallel with a blank. Each series of measurements was repeated 3 times on different days, each time with freshly prepared potato suspensions. Thus, all reported results of measurements are mean values of 9 data.

1.2.3. *Mathematical statistical evaluation of the results.* Student's *t* test was used to compare the results of measurements (KÖRMENDY, 1964). Regression equations and correlation coefficients were calculated in the usual way.

## 2. Results

### 2.1. Selection of the wave length to determine optical density

Reaction mixtures of 0.8% w/v substrate content and pH 6.2 were incubated for 0, 30, 60, 90, 120, 150 and 180 minutes, respectively, to establish an appropriate wave length for further measurements. Optical density readings of the filtrates of the mixtures were taken at the end of the incubation period at every 25 nm between 350 and 500 nm. At wave lengths below 350 nm zeroing of the instrument was not possible, above 550 nm very low optical density values were obtained which decreased further with increasing wave lengths.

The results are illustrated in Fig. 1.

It can be seen from the figure that with increasing reaction time absorption maxima shift towards higher wave lengths.

On the basis of absorption maxima the wave length range of 375–425 nm seemed suitable for measurement. Within these limits the appropriate wave length for the measurements was chosen on the basis of the relationship between optical density and reaction time.

In the reaction time interval between 0 and 150 minutes, the quickest change — as can be seen from Fig. 2 — was obtained at 400 and 425 nm, therefore these two wave lengths are the most favourable as regards the sensitivity of measurement.

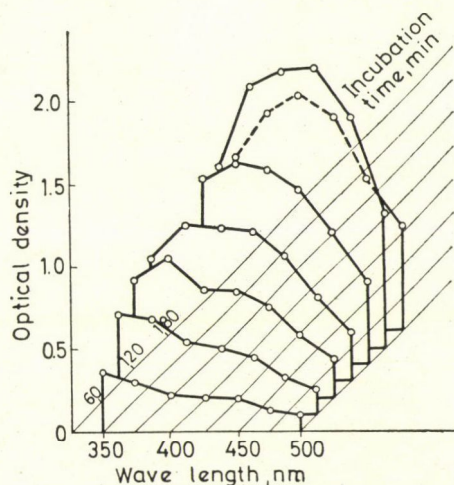


Fig. 1. Optical density plotted against wave length and reaction time, using a substrate concentration of 0.8% w/v



At 0.8 w/v substrate concentration and in the interval between 0 and 90 minutes, optical density at these two wave lengths is in linear relationship with reaction time. This, too, is advantageous from the point of view of activity measurements. The respective regression equations are

$$y = 0.24 + 0.007x \text{ and}$$

$$y = 0.19 + 0.008x,$$

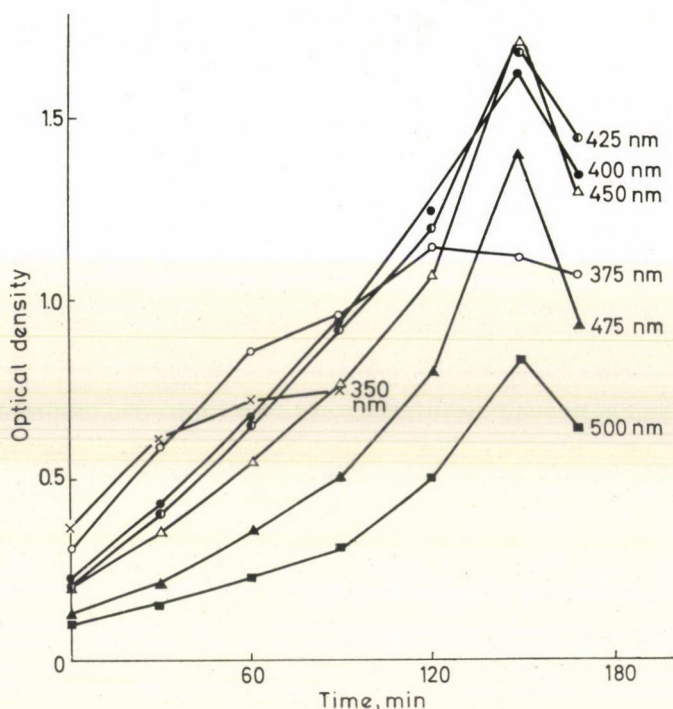


Fig. 2. Changes in optical density with reaction time at different wave lengths, using a substrate concentration of 0.8% w/v

where  $x$  = the reaction time in minutes and

$y$  = the extinction modulus.

The correlation coefficients are 0.95 and 0.96, resp.

(For 375 nm, the slope of the straight section, as expressed by the regression coefficient, is somewhat higher:

$$y = 0.37 + 0.009x \quad (r = 0.92),$$

but linearity holds only from 0 to 60 min, therefore this wave length was ruled out.)

The values obtained at different times of measurement are, in the linear section (ranging from 0 to 90 minutes), significantly or highly significantly different from each other, therefore this range is suitable for measurement. The optical density readings taken at 400 and 425 nm after the same incubation periods do not differ significantly from each other (Fig. 1) (no significant difference exists between the two regression coefficients), thus both wave lengths are equally suitable for measurement. 400 nm was chosen for further work.

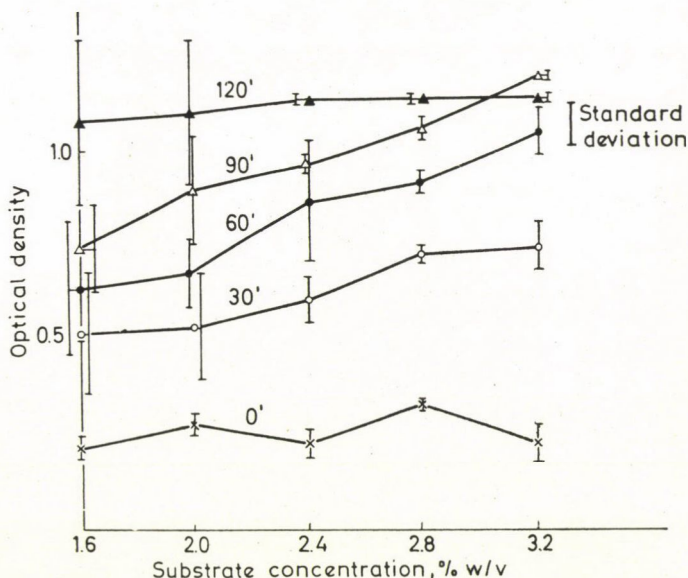


Fig. 3. Changes in optical density at 400 nm with substrate concentration at different reaction times

## 2.2. Determination of optimum substrate concentration

When using a 0.8% w/v substrate, the optical density of the reaction mixture decreased significantly at most of the wave lengths in the reaction time interval between 150 and 180 minutes (Fig. 2). This indicates depletion of the substrate or occurrence of secondary reactions causing decrease in optical density.

Experiments were carried out with increased substrate concentrations to ensure substrate saturation: optical density readings of the reaction mixtures were taken every 30 minutes in the interval between 0 and 120 minutes, with substrate concentrations ranging from 1.6 to 3.2% w/v.

The results are presented in Fig. 3.

It can be seen from the figure that the standard deviations of the measurements are fairly high: the average variation coefficient is 11.3%.



The initial optical density of the reaction mixture is practically the same for all the substrate concentrations tested above 1.6% w/v: readings of the optical densities with 1.6, 2.4 and 3.2% w/v substrate taken at 0 time did not differ significantly from one another. The optical densities measured after reaction times different from 0 increased in general with substrate concentra-

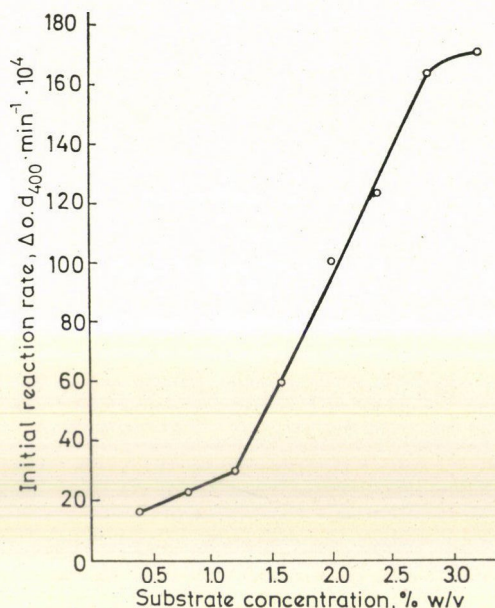


Fig. 4. Initial reaction rate as calculated from optical density changes at 400 nm plotted against substrate concentration

tion, with the exception of those taken after 60 minutes, which did not change significantly in the concentration range from 1.6 to 3.2% w/v. No significant difference was found either between the values obtained after 30 and 60 minutes, respectively, with substrate concentrations of 2.8 and 3.2% w/v.

Thus, further measurements were carried out with 3.2% w/v substrate concentration and 0–60 minutes reaction time.

Fig. 4, in which initial reaction rates as calculated from optical density readings taken at 0 and 30 minutes, are plotted against substrate concentration, justifies this choice, for the increase in ordinate values is markedly slowed down in the substrate concentration range above 2.8 w/v.

On applying the above substrate concentration, optical density of the blank increases on incubation at pH 6.2 and 30°C in the interval between 0 and 60 minutes proportionally to time, in accordance with the regression equation  $y = 0.16 + 0.0044x$  (the correlation coefficient is 0.98;  $x$  = reaction time and  $y$  = o.d.).

### 2.3. Determination of initial reaction rates

In order to determine initial reaction rates, a 3.2% w/v substrate was used and optical density readings of the reaction mixture were taken every 10 minutes in the reaction time interval between 0 and 60 minutes. The results are shown in Fig. 5.

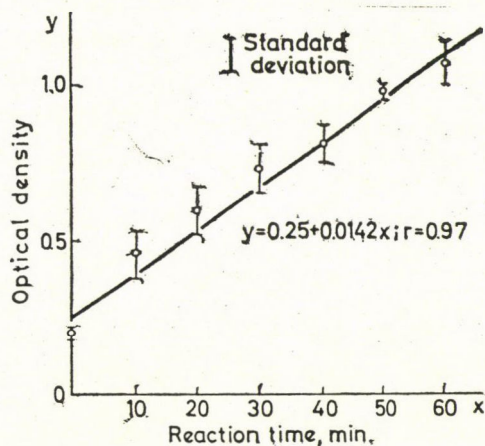


Fig. 5. Changes in optical density at 400 nm with reaction time, using a substrate concentration of 3.2% w/v at pH 6.2

In the 0 to 60-minute interval the increase in o.d. as a function of reaction time may be considered constant (the correlation coefficient of the regression equation is 0.97).

The constant of the regression equation is 0.25, thus the reaction mixture has measurable optical density even at the so-called "0" time. The major part of this optical density may be attributed to the substrate, which, at the actual "0" time, has a corresponding value of 0.16 (para. 2.2). The difference of the two values, 0.09 optical density may be ascribed to the fact that, owing to hand pipetting, a lapse of about 3 minutes occurs between the preparation of the reaction mixture and the "0" time measurement. During this time no shaking of the reaction mixture takes place. Therefore, colour formation which may be observed directly upon mixing of the components, is probably due to the oxygen absorbed by the solutions in the course of preparation, in spite of the use of boiled distilled water. The majority of dissolved oxygen is, probably, due to homogenization in air in the Atomix.

### 2.4. Effect of enzyme concentration on reaction rate

Enzyme concentration in the reaction mixture was varied by decreasing potato concentration (para. 1.2.1). The optical density values of the reaction



mixtures of 0.5—2.0% w/v potato content in the reaction time interval between 0 and 60 minutes are represented in Fig. 6.

It can be seen from the figure that — between the given limits — optical density is in linear relationship with the potato content, thus with enzyme concentration.

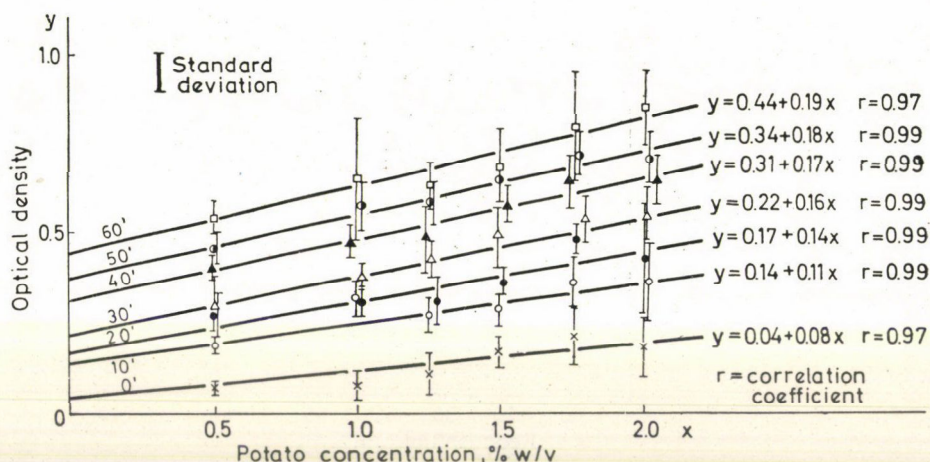


Fig. 6. Changes in optical density at 400 nm with potato concentration after different reaction periods

However, since the curves do not go through the origo, it is not possible to deduce, solely from the optical density values obtained at a certain reaction time with a given potato (enzyme) concentration, the corresponding values for other potato (enzyme) concentrations. Such calculation requires the knowledge of the equation of the curve.

With different varieties the same potato concentration may correspond to different enzyme concentrations, consequently, the resulting error has to be taken into consideration, too.

In order to study this possible error, the optical density readings from Fig. 6 were plotted against reaction time. The relationship between reaction time and the optical densities of the reaction mixtures of different potato content may be characterized by the linear regression equations given in Table 1, in which the reaction rates are expressed by the coefficients of  $x$ .

On relating the reaction rates to 1 g potato, with increasing potato concentration of the reaction mixture decreasing values are obtained. The relationship can be described by a hyperbola (Fig. 7) corresponding to the following equation:

$$y = \left( 130 + \frac{713}{c} \right) 10^{-4}.$$

Table 1

*Linear regression equations expressing the changes in optical density with time of reaction mixtures of different potato content*

Substrate concentration: 3.2% w/v, reaction time: 0–60 min, pH 6.2

Sampling: every 10 minutes

Potato concentration, % w/v	Linear regression equation of change in o.d.	Correlation coefficients of the equations (r)	Rate of change in o.d. $E \cdot \text{min}^{-1} \cdot 10^4$	Rate of change in o.d. related to 1 g potato $E \cdot \text{min}^{-1} \cdot \text{g}^{-1} \cdot 10^4$
0.50	$y = 0.090 + 0.0077x$	0.99	77	1 540
1.00	$y = 0.092 + 0.0086x$	0.99	86	860
1.25	$y = 0.127 + 0.0087x$	0.99	87	696
1.50	$y = 0.139 + 0.0087x$	0.99	87	580
1.75	$y = 0.245 + 0.0095x$	0.99	95	543
2.00	$y = 0.220 + 0.0103x$	0.99	103	515

$x$  = reaction time, min

$y$  = optical density (o.d.)

$E$  = o.d. modulus

Accordingly, the reaction rate related to unit amount of potato, expressing the enzyme concentration, depends on potato concentration. However, due to the hyperbolic character of the relationship — as can be seen also from Fig. 7 — the higher the potato concentration of the reaction mixture the less significant is the change of the enzyme concentration as a function of potato content.

## 2.5. Influence of pH on the reaction rate

Although the potato has a pH of about 6.0 and thus in practice measurement at pH 6.2 is the most suitable, for the sake of completeness, additional measurements were carried out at the following pH values: 5.0, 6.7 and 7.3. At pH 5.0 the enzyme was not active. The relationship between optical density and reaction time, as established at the other two pH values, are shown in Fig. 8. In these two series of measurements optical density readings were taken every 5 minutes in the reaction time interval between 0 and 30 and 0 and 20 minutes, respectively. For the sake of comparison, the section of the curve between 0 and 30 minutes established at pH 6.2 is also represented in the figure.

The optical density value measured at pH 7.3 after 20 minutes was not taken into consideration in calculating the regression line, as — probably due to the depletion of the substrate — at this time the correlation extensively deviated from the linear.



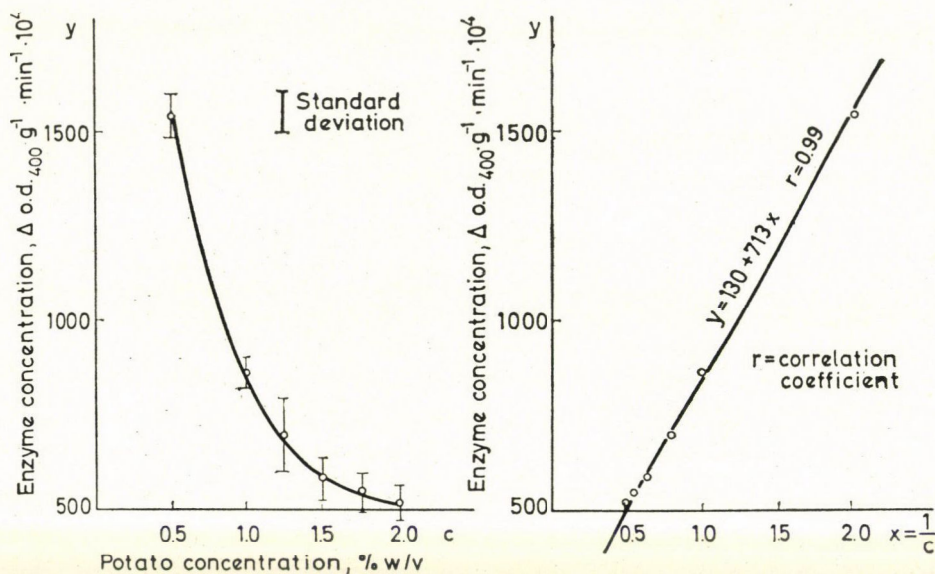


Fig. 7. The rate of change in optical density at 400 nm related to 1 g of potato plotted against potato concentration and the regression curve of the reciprocal correlation

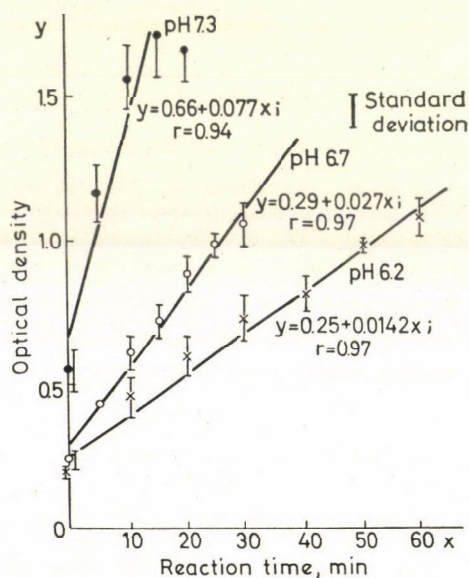


Fig. 8. Changes in optical density at 400 nm with reaction time in reaction mixtures of different pH values, using a substrate concentration of 3.2% w/v

The reaction rate increases with increasing pH: at pH 6.2 the regression coefficient is 0.014, at pH 6.7 nearly the double, 0.027, while at pH 7.3 its value is more than five times higher, 0.077. The increase in reaction rate is more pronounced between pH 6.7 and 7.3 than between pH 6.2 and 6.7. The comparison of the adjacent pairs of curves showed a very highly significant difference of the regression coefficients (pH 6.2 vs. pH 6.7:  $DF = 10$ ;  $t = 7.59$  and pH 6.7 vs. pH 7.3:  $DF = 9$ ,  $t = 42.27$ ).

## 2.6. Study of the necessity of enzyme inactivation

In general, at the end of the reaction time the enzyme has to be inactivated to prevent the occurrence of further changes until measurement is carried out. The oxidizing action of ODO requires molecular oxygen, therefore it is presumable that after shaking has been stopped the reaction may take place only on the surface of the mixture which is in contact with air (in the cuvettes of the spectrophotometer this surface is 1 cm<sup>2</sup>, which is negligible in comparison to the volume of 5 ml). In spite of that it was thought advisable to examine if the optical density of the reaction mixture would change on longer standing without aeration.

Therefore, the optical density of a pH 6.2 reaction mixture previously incubated for 1 hour was measured at 400 nm immediately after filtration and after 3 hours of storage in the refrigerator at +5°C. Simultaneously in a second similar reaction mixture the enzyme was inactivated at the end of the reaction period by 1 drop of cc. HCl. Optical density of this mixture was measured, (a) immediately and (b) after 3 hours of storage in the refrigerator. The results are shown in Table 2.

It is seen from the table that on storage for 3 hours in the refrigerator the optical density of the reaction mixture does not change significantly. Since, in general, readings may be taken in much less time, it is not necessary to inactivate the enzyme at the end of the reaction period. Hydrochloric acid treatment alters the optical density of the reaction mixture highly significantly. The optical density value thus obtained does not undergo significant changes either, on further storage at +5°C ( $DF = 16$ ,  $t = 1.39$ ).

## 2.7. Method developed for activity measurement and definition of the unit of ODO activity

On the basis of the results of the experiments the method described as follows was found suitable for the measurement of potato ODO activity:

3 potato tubers are peeled and cut into small cubes with a stainless steel knife. The diced potatoes are kept under water until further comminution. 10-g portions of the potatoes dried on the surface by filter paper are homog-



Table 2

*Effect of storage and acid inactivation upon  
the optical density of the reaction mixture*

Treatment of the reaction mixture	Optical density		Significance level of the differences in optical densities*
	mean	standard deviation	
1. Measured immediately at the end of the reaction period	0.74	0.14	
2. Measured after a 3-hour storage at +5°C following the reaction period	0.77	0.11	Ø
3. Measured at the end of the reaction period, after addition of 1 drop of cc. HCl	0.64	0.12	xx
4. Measured after a 3-hour storage at +5°C following addition of 1 drop of cc. HCl at the end of the reaction period	0.73	0.14	Ø

*Conditions of experiment:* Substrate concentration: 0.4% w/v; reaction period: 1 hour; pH: 6.2.

\*The optical densities of the reaction mixtures treated in different ways were compared to the o.d. of the mixture measured immediately at the end of the reaction period (Experiment No. 1). Ø: non significant, xx: highly significant

enized for 3.0 minutes with 90 ml of 0.1 *M*, pH 4.0 citrate buffer (13.1 ml of 0.1 *M* citric acid + 6.9 ml of 0.1 *M* trisodium citrate solution) in an Atomix homogenizer equipped with knives.

A 4.0% w/v pyrogallol solution, freshly prepared daily, is used as substrate. Pyrogallol is dissolved in a 0.1 *M*, pH 7.0 phosphate buffer (61.0 ml of 0.2 *M* Na<sub>2</sub>HPO<sub>4</sub> + 39.0 ml of 0.2 *M* NaH<sub>2</sub>PO<sub>4</sub> solution, made up with distilled water to 200 ml).

8 ml of the buffered substrate solution preheated to 30°C are mixed with 2 ml of the enzyme suspension (the pH of the reaction mixture is 6.2–6.4).

The mixture of 8 ml of substrate and 2 ml of pH 4.0 buffer is used as blank. The pH values of the blank and of the reaction mixture have to be strictly identical.

4 parallels are run of the blank and 12 of the reaction mixture containing the enzyme. The mixtures are incubated for periods of 0–30 or 0–60 minutes at 30°C under shaking (0.036 mmole O<sub>2</sub>/min) in 50 ml open wide-necked Erlenmeyer flasks placed in the water bath "Vibroterm" (manufactured by Labor-MIM, Hungary). The first sample is taken immediately on pouring together the components of the reaction mixture. Further on, 1 blank and 3 samples of the reaction mixture are removed from the water bath every 10 or 20 minutes, respectively. The samples are filtered through folded filter paper prepared in advance and the optical densities of the filtrates are determined at 400 nm in a MOM 203 spectrophotometer against the blanks incubated for identical periods.

The arithmetical means of the 3 parallel measurements are considered as the optical density values corresponding to the different incubation periods. The measurement is acceptable if the average variation coefficient does not exceed 10 to 12%. (If otherwise, it must be assumed that pipetting of the inhomogeneous potato suspension was not carried out with sufficient accuracy.) Plotting optical densities against reaction time, a straight line has to be obtained, the characteristics of which may be determined by linear regression.

The degree of enzyme activity is indicated by the slope of the regression line, *i.e.* by the coefficient of  $x$  in the equation.

Activity is defined as the change in optical density occurring during 1 minute under the given conditions. The enzyme is considered as being of unit activity if the change, brought about by its action, in the optical density modulus is  $1 \cdot 10^{-4}/\text{min}$ .

From the equation of the curve in Fig. 4, the value of ODO activity of the potato variety "Gül baba" is 142 U. Since the reaction mixture contained 0.2 g of potatoes, the enzyme concentration is  $710 \text{ U} \cdot \text{g}^{-1}$  of potato tissue.

Quick information may be gained by measuring the optical density, beside zero, only at one more reaction time between 0 and 60 minutes and calculating enzyme activity from the equation of the curve determined by these two mean values. This is, however, not advisable if comminution of the potatoes is carried out with the equipment described which yields a rough suspension with quickly settling particles. The error, caused by unequal particle distribution in the reaction mixtures, may be reduced by calculating activity values from several points of measurement.

### 3. Conclusions

The relatively quick, simple and well reproducible method developed is, according to experience, suitable for the determination of ODO activity in potatoes.

As seen from Fig. 8, the enzyme reaction may be accelerated by increasing the pH from 6.2 to 7.3, increasing thereby the sensitivity of the measurement. In spite of that, pH 6.2 was chosen for the enzyme reaction. One of the reasons in doing so was that — of all the values investigated — this one is nearest to the pH of the potato itself, another, that substrate stability is highest at this pH. In more alkaline media pyrogallol is easily oxidized in a non-enzymatic way, furthermore insoluble dark polymers are soon formed which may falsify the results of the optical density measurements. Therefore it is probable that at higher pH values it would become necessary to inactivate the enzyme at the end of the reaction time (para. 2.6).



The colour formed instantly on combining the components of the reaction mixture (deeper than the colour of pyrogallol proper), is also stronger at higher pH values. This colour, however, does not, according to definition, influence the activity values measured. The pH-dependence of the initial colour of the reaction mixture made it necessary to use, contrary to general practice, enzyme-free substrate as blank at a pH identical with that of the reaction mixture. Heat inactivation of the enzyme would have brought about starch paste formation resulting in poor filtrability, while acid inactivation would have altered both colour and optical density of the blank similarly to the acid treatment of the incubated reaction mixture which had caused significant changes in optical density as compared with that of the non-inactivated sample (Table 2).

The part of the initial optical density of the reaction mixture which does not originate from the substrate (actually only 0.09 o.d. units, since, according to the equation, after 3 minutes the optical density of the substrate is 0.16) was not taken into correction, as this was formed under conditions of oxygen supply different from those subsisting during incubation, and furthermore, practically uncontrollable. Aeration by shaking starts, on the other hand, at the so-called "0" time, *i.e.* after a lapse of 3 minutes. If fewer samples are placed at the same time into the water bath "Vibroterm", the error caused by manipulation may be considerably reduced. This is of importance in determining kinetic constants. In routine analysis the relative error due to manipulation may be reduced by employing suitable (longer) reaction periods. Optical density, as calculated from the equation of the curve in Fig. 5, is 0.67 after 30 and 0.84 after 60 minutes, the error caused by manipulation constitutes 12 and 10%, resp., of these values. Considering the standard deviations this error is within the accuracy limits of the method. As the relationship between optical density and reaction time is linear in the interval between 0 and 60 minutes, longer reaction periods within this interval do not affect the value of the activity.

The activity unit defined is arbitrary and deviates from the principles given by the International Union of Biochemistry, (ANON, 1965) in as much, as instead of micromoles of the substrate transformed per minute, it takes as a basis the change in optical density per minute. ODO action results in a series of products of different degrees of oxidation and polymerization which cannot be easily removed from the reaction mixture and make substrate determination difficult. On the other hand, the choice of an arbitrary unit does not restrict the practical applicability of the method.

The methodics applied allow to determine but the cell-bound enzyme activity, whereas soluble ODO gets lost, from the point of view of the assay, during storage under water of the diced potato. Although, according to the literature (VÁMOS & BALÁZS, 1970) only a negligible part of the enzyme is



present in soluble form in horticultural products, it seems indicated to extend the method to assessing the activity of the latter, too, as the ratio of soluble and cell-bound enzyme activities may be characteristic of the different varieties at given stages of maturity.

Finally, a method ought to be developed for the numerical definition of the correlation between the browning tendency of potatoes and the values obtained by activity measurement. Experiments to this effect with several potato varieties have been considered.

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## APPLICATION OF A METHOD OF THIN-LAYER CHROMATOGRAPHY TO THE DETERMINATION OF CITRIC ACID IN POTATOES

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A method of thin-layer chromatography was developed for the determination of citric acid in potatoes. Citric acid was extracted during a 2-hour period from the powdered potatoes. Kieselgel G was used as adsorbent and a mixture of ethyl acetate-formic acid-distilled water (3:1:1) as developing solvent. The development took 80 minutes as against the 7 hours time requirement of development in paper chromatography. The citric acid spots were detected with the methyl alcohol solution of bromophenol blue and methyl red. The spots are orange in colour on a blue background.

Quantitative evaluation was carried out by a panel of judges on the basis of comparing the spots obtained from potato extract to spots obtained from a series of standard citric acid solutions. Fig. 1 shows the spots corresponding to various amounts of pure citric acid. Fig. 2 shows the spots developed from various amounts of potato extract parallel to those corresponding to various amounts of citric acid. Results obtained by visual evaluation are given in Table 1. The table contains the average values obtained by quantitative evaluation of various amounts of potato extract, the standard deviations and coefficients of variation.

The coefficient of variation of the method described was 3%.

Recently increasing attention is being paid to the quantitative determination of organic acids in the chemical analysis of raw materials of plant origin. The amount of organic acids present is not only a physiological quality characteristic but may affect the suitability to processing due to reactions of the acids with trace elements present in the raw material. The enrichment of the material in organic acids is usually followed by the improvement of colour. On the other hand, for instance in potatoes, the reduction or absence of organic acids may be one of the reasons of non-enzymic browning.

According to relevant literature the blackening of cooked potatoes on standing may be attributed to citric acid. This was supported by the findings of HEISLER and co-workers (1964), who carried out a study of organic acids in 41 potato samples and found that citric acid was the major component, amounting to about 50% of the total amount of acids.

Thus in the course of our work we wished to develop a method for the detection and quantitative determination of citric acid.

In the choice of the most suitable method the methods described in the literature were taken into account and some of them were tested. The quantitative methods found were based on different principles. PUCHER and co-wor-



kers (1936), TAUSSKY and SHORR (1947), RUTTLOFF and BEHNKE (1957), MACDONALD and WATERBURY (1959), HARTFORD (1962) have given methods based on colorimetry. BOSER's method (1955) utilized the complex forming capacity of citric acid with copper. SCHWARTZ and co-workers (1962) developed a column chromatography method and used an anion exchange resin as an adsorbent and fractionated elution. The fractions were then titrated with an alkaline solution. The same method was applied by HEISLER and co-workers (1964) in their study of the role of citric acid in the blackening of potatoes. JURICS and LINDNER (1965) determined citric acid in potatoes by paper chromatography. However, the time requirement of all of the methods enumerated is extensive. Even the shorter version of the paper chromatography method required 7 hours development time.

After various preliminary experiments thin-layer chromatography, widely used at present in chemical analysis, was applied. BRAUN and GEENEN (1962), STAHL (1962), BANCHER and SCHERZ (1964), among others, separated carboxylic acids and hydroxy acids by thin-layer chromatography.

## 1. Materials and methods

Ten samples, true to variety, were used to develop a method for the determination of citric acid in potatoes.

The study was carried out in two stages. First pure citric acid was determined by thin-layer chromatography and then the method developed was applied to the quantitative determination of citric acid in potatoes.

### 1.1. Determination of citric acid in pure solution

The aim of these studies was to select the most suitable adsorbent and developing solvent. After several trials the adsorbent Kieselgel G (Merck) and the developing solvent ethyl acetate-formic acid-distilled water (3 : 1 : 1) as suggested by BANCHER and SCHERZ (1964), was found the most suitable. A mixture of 0.3 g bromophenol blue and 0.1 g methyl red dissolved in 100 ml methyl alcohol, was used to detect the spots. The standard solution was prepared by dissolving 0.1% citric acid in ether.

20 × 20 cm glass plates were covered with a Kieselgel G layer of 0.25 mm thickness. The plates were then oven-dried for 10 minutes at 105°C and used immediately.

0.1–0.8  $\mu$ l of the standard solution was applied as test spot. A mixture of ethyl acetate-formic acid-distilled water was used for developing the spots. Time of development was 80 minutes. The distance of the solvent front from the start line was 11 cm, the R<sub>f</sub>-value 0.70.



Prior to detection the thin-layer had been dried for 10 minutes and then sprayed with the spray reagent. Clearly distinguishable spots appear if the layer is dried in warm air (about 60—70°C).

### 1.2. Thin-layer chromatography of the citric acid content of potatoes

Various extraction methods (using water, methanol, ether) were applied in order to increase accuracy of spotting and to liberate bound citric acid.

1.2.1. *Extraction with ether.* As a conclusion of preliminary experiments ether was used for extraction, because organic acids dissolve easily in this solvent (PAECH & TRACEY, 1956). A further advantage of using ether is that it can be better applied to the adsorbent than an aqueous or methanol solution. Citric acid present in salt-form may be liberated by strong inorganic acids while these are practically insoluble in ether.

The potatoes were sliced and the slices dried under infra-red lamps. The dried slices were powdered. The exact amount of 2.5 g was weighed and ground with 20 g anhydrous  $\text{Na}_2\text{SO}_4$  and 20 g analytical grade quartz sand. To liberate bound citric acid 2.5 ml diluted sulfuric acid (10 ml cc.  $\text{H}_2\text{SO}_4$  — 70 ml distilled water) was added and grinding continued. The sample thus prepared was transferred to an extraction thimble. Extraction was carried on in a Soxhlet apparatus for 2 hours, with about 200 ml ethyl ether. When extraction was finished the extract was adjusted to 50 ml. Excess ethyl ether was evaporated under vacuum. The solution thus obtained was considered the stock solution and used for the determination of the total acid content by titration with 0.1 N NaOH and the citric acid content by thin-layer chromatography.

1.2.2. *Thin-layer chromatography.* The extract, evaporated to a pre-determined volume, was applied with the aid of a micropipette to the thin-layer. The adsorbent used was either Kieselgel G (Merck) as described in para. 1.1. or pre-coated Polygram SIL G (Macherey-Nagel). The adsorbent is the same in both preparations, only in the pre-coated layer it is bound more firmly and therefore less liable to damage and of better storage stability. The start line was two cm from the lower edge of the plate and spots were applied at 2 cm intervals, six altogether (numbered 1 to 6). The application of the sample to be studied and of the standards was carried out according to the method of BLAZOVICH *et al.* (1969). According to this method the 0.1% standard solution and the potato extract of unknown concentration were applied in increasing quantities thereby permitting of the visual judgement of the citric acid concentration of the unknown solution.

According to these authors visual evaluation of the spots is vastly improved if the diameter of the test spots as applied is not larger than 5—6 mm, and the volume applied of the standard solutions and of the extract is approximately the same and not more than 0.1 ml.

Application of the spots was as follows: at test spots Nos. 1, 3 and 5, 15,



20, 25  $\mu$ l, respectively, of the 0.1% standard citric acid solution and at Nos. 2, 4, 6 the sample investigated. It is expedient to apply of the sample investigated' an amount the citric acid content of which approximates that of the standard solution next to it.

Development and visualization was carried out as described in para. 1.1. After the application of the spray reagent citric acid appeared as orange spots on a blue background.

## 2. Results

The thin-layer chromatogram of the standard solution as given in para. 1.1. is shown in Fig. 1.

The figure illustrates the spots of 0.1% citric acid solution in ether. The solution was applied in amounts increasing by 10  $\mu$ g. As is seen, the size of the spots and thus the quantity of citric acid can be differentiated. The first spot, containing 10  $\mu$ g of citric acid is somewhat difficult to evaluate, however, the next spot containing 20  $\mu$ g of citric acid and subsequent spots containing higher amounts can easily be identified.

Fig. 2 shows a chromatogram of the standard citric acid solution and of the potato extract of unknown citric acid content.

To start points Nos. 1, 3, and 5, 15, 20, 25  $\mu$ l, respectively, of the 0.1% citric acid solution (containing 15, 20, 25  $\mu$ g, citric acid, respectively) were applied. To start points Nos. 2, 4, 6 the potato extract was applied in the same volumes.

As seen in the figure the size of the spots pertaining to the potato extract is between the size of the spots pertaining to the standard solutions on either side. Spot No. 2 developed from 15  $\mu$ l potato extract (2.5 g powdered potatoes in 50 ml ethyl ether) is somewhat larger than the spot developed from 15  $\mu$ g citric acid (No. 1) and somewhat smaller than the one developed from 20  $\mu$ g citric acid (No. 3). Similarly the size of the spot developed from 20  $\mu$ l potato extract (No. 4) is larger than the spot developed from 20  $\mu$ g standard solution and smaller than the one developed from 25  $\mu$ g citric acid (Nos. 3 and 5, resp.).

The results of visual quantitative evaluation pertaining to 5 potato varieties, are given in Table 1.

Data were obtained by the visual evaluation of the spots developed from three different extract quantities of individual potato samples applied along with known amounts of the standard solution. Evaluation was carried out always by the same five panelists. Variation of the results was investigated by analysis of variance.

Four hours are required to carry out a determination complete with preparatory work.

In the 5 potato varieties investigated an average citric acid content of 1.91–0.3 g/100 g dry matter was found.



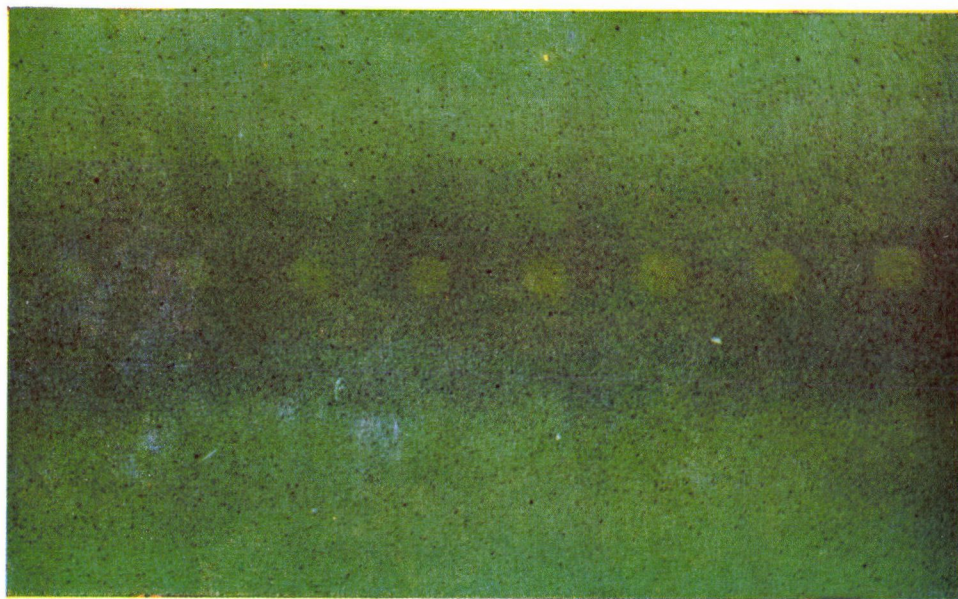


Fig. 1. Standard solutions of citric acid. Amounts of standard citric acid solution applied: 0.01; 0.02; 0.03 ... 0.08 ml corresponding to 10; 20; 30; ... 80  $\mu\text{g}$  citric acid. Layer: Kieselgel G (Merck). Solvent: ethyl acetate-formic acid-distilled water (3:1:1). Colour reagent: 0.33 g bromophenol blue and 0.1 g methyl red dissolved in 100 ml methyl alcohol. Colour of the spots: orange on blue background. Distance of solvent front from start line: 11 cm, Rf: 0.70

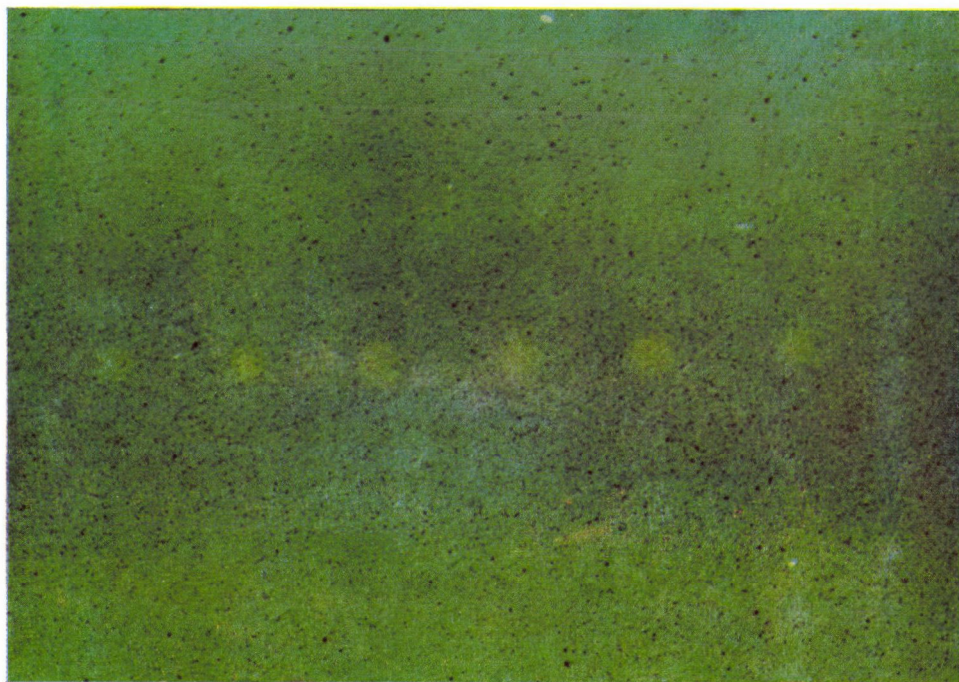


Fig. 2. Thin-layer chromatogram of potato sample No. 1. Amounts applied: (from left to right): spot No. 1: 15  $\mu\text{g}$  citric acid, spot No. 2: 15  $\mu\text{l}$  potato extract, spot No. 3: 20  $\mu\text{g}$  citric acid, spot No. 4: 20  $\mu\text{l}$  potato extract, spot No. 5: 25  $\mu\text{g}$  citric acid, spot No. 6: 25  $\mu\text{l}$  potato extract. Layer: Kieselgel G (Merck). Solvent: ethyl acetate-formic acid-distilled water (3:1:1). Colour reagent: 0.3 g bromophenol blue and 0.1 g methyl red dissolved in 100 ml methyl alcohol. Colour of the spots: orange on blue background. Distance of solvent front from start line: 11 cm, Rf: 0.70



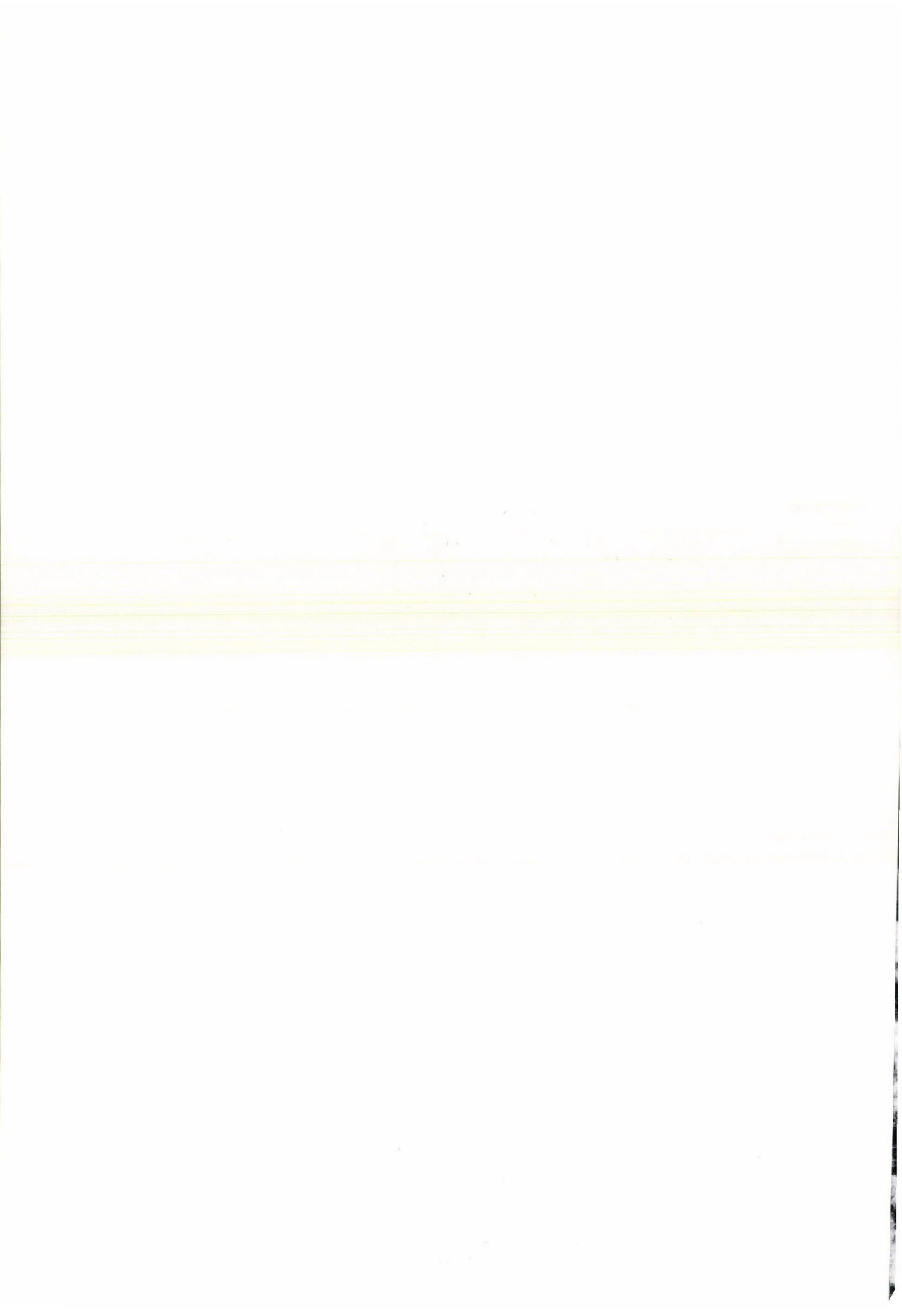


Table 1

*Quantitative thin-layer chromatographic determination of citric acid in potatoes*

Layer: Kieselgel G (Merck)

Solvent: ethyl acetate-formic acid-distilled water (3 : 1 : 1)

Colour reagent: 0.3 g bromophenol blue and 0.1 g methyl red dissolved in 100 ml ethyl alcohol

Distance of the solvent front from the start line: 11 cm

Rf: 0.70

Sample	Quantity of extract applied	Average result of 5 panelists	Standard deviation	Coefficient of variation
	$\mu\text{l}$	$\mu\text{g}$	s	v%
1.	15	17.4	0.90	5.2
	20	22.8	1.30	5.7
	25	27.2	0.14	0.5
2.	15	11.6	0.48	4.1
	20	16.0	1.00	6.2
	25	20.0	1.20	6.0
3.	15	15.2	0.44	2.8
	20	21.2	1.09	5.1
	25	25.6	0.54	2.1
4.	15	13.6	1.82	4.0
	20	18.0	0.70	4.0
	25	22.2	1.92	8.6
5.	15	14.4	1.15	7.9
	20	18.8	1.64	8.7
	25	23.8	1.30	5.4

### 3. Conclusions

The method developed seems suitable for the determination of the citric acid content of potato tubers.

The extraction of the organic acid content of potatoes with ethyl ether is simple and rapid. The total organic acid content can be determined by titration of the extract. After evaporation of the extract under vacuum the citric acid content can be determined by thin-layer chromatography. The advantage of the method lies in its low time requirement. Since the spots developed have sharp contours, evaluation can be carried out with the required accuracy. Beside the determination of the citric acid content by thin-layer chromatography the total acid content can also be determined from the ether extract.



The method seems suitable for the determination of citric acid in other raw materials of vegetable origin.

\*

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## EFFECT OF GAMMA RADIATION ON THE VIABLE CELL COUNT AND SOME OTHER QUALITY CHARACTERISTICS OF DRIED ONIONS

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The results of experiments carried out with onion powder and flakes of Egyptian origin show a radiation treatment of 0.1—0.2 Mrad sufficient to reduce the microbial contamination of dried onions by one order of magnitude. To achieve a 99% reduction, satisfactory from the practical point of view, treatment with 0.5—0.6 Mrad seems necessary.

With increasing radiation doses the characteristic smell of dried onions diminished and a caramel-like off-odour appeared and the colour of the dried products darkened. These changes become significant only above treatment with 0.8 Mrad. The differences between radiation treated and untreated samples both packed in Cellothene pouches were either eliminated (odour) or diminished (colour) during 6 weeks storage following irradiation.

The results obtained by the chemical oxygen demand method (COD) based on bichromate oxidation of water soluble volatile reducing substances, were not in accord with the organoleptically observable changes of odour.

Growing interest is shown in dried onions both by the housewife and in the food industry. Since in a number of cases, for instance in salads, sauces and several meat products the onions are not exposed to heat treatment during rehydration, or are subjected to a very mild treatment only, it is most important to obtain dried onions of very low microbial contamination. However, in spite of the most careful drying operations the viable cell count of dried onions is frequently substantial. Thus it seems of interest to investigate the possibilities of reducing the viable cell count. In the knowledge of the bactericidal effect of ionizing radiations it seems worth while to use this physical procedure to reduce the cell count of dried products. The present experiments were aimed at reducing the viable cell count of dried onions with ionizing radiation and various characteristics of the untreated and treated samples were compared.

In this preliminary study un-inoculated dried onion was used in order to establish the radiation sensitivity of the "natural" microbial flora of the product.

### 1. Materials and methods

In the experiments onion powder and onion flakes of Egyptian origin were used.



20 g of the powder and 10 g of the flakes were packed in Cellothene\* pouches of 70  $\mu\text{m}$  wall thickness and irradiated, then stored at ambient temperature till used.

Irradiation was carried out with an LMB-gamma-1 type self-shielded laboratory gamma radiation source (dose rate: 0.28 Mrad/hour). The dose was determined with the Fricke ferrous sulfate chemical dosimeter.

The onion flakes were ground to powder under aseptical conditions prior to chemical and microbiological tests.

The total viable aerobic cell count and the mould count were determined by plating, on universal agar medium\*\* and incubation at 30°C. To determine the coliform cell count the modified agar medium of MacConkey (Oxoid Manual, 1967, p. 175) was used and the cultures were incubated at 37°C.

The number of colonies was established after an incubation period of 48 hours. A sterile diluting liquor containing 0.1% peptone was used for the decimal dilutions in the viable cell count determination.

To estimate the water soluble volatile reducing substances the method of SAGUY and co-workers (1970), based on the chemical oxygen demand (COD) was used.

A Perkin—Elmer, type UV 137 spectrophotometer served for optical density measurement.

For the sensory evaluation of the colour and odour of the dry samples KRAMER's ranking method was applied (KRAMER, 1960).

## 2. Results

The results of viable cell count determinations are summarized in Tables 1 and 2.

To establish the *proneness to deterioration* of the rehydrated onion 2-g samples of the untreated and radiation treated material were suspended in 30 ml distilled water each and were shaken in an apparatus provided with water bath at 30°C for 24 hours. The untreated sample and that treated with 0.1 Mrad was spoiled and emitted offensive putrid smell. On the sample treated with 0.2 Mrad off-odour was observed, a sign of commencing spoilage, while the samples treated with 0.8 and 1.6 Mrad, resp., were unchanged. The filtrate of the samples obtained by filtering through G-3 sintered glass filter showed increasing turbidity with increasing radiation dose, therefore the optical density was measured at 650 nm on the spectrophotometer. The results are shown in Table 3.

\* Cellothene = 20  $\mu\text{m}$  thick cellophane coated on the inside with low density polyethylene (50  $\mu\text{m}$ ).

\*\* Composition: 200 ml sweet whey, 100 ml yeast extract (1:10), 4 g meat extract, 2 g peptone, 10 g glucose, 20 g agar-agar, 700 ml water. The medium was adjusted to pH = 7.2 and was sterilized at 2 at gauge pressure.

Table 1

*Reduction of viable cell count in powdered onion as a result of gamma irradiation*

Radiation dose (Mrad)	Total viable aerobic cell count/g	Reduction of total viable cell count (%)	Mould count/g	Reduction of mould count (%)	Cell count of coliform bacteria/g	Reduction of coliform cell count (%)
0	6 200	—	2 500	—	19	—
0.1	690	88.8	170	93.2	14	26.3
0.2	350	94.3	6	99.8	6	68.4
0.8	38	99.4	< 1	> 99.96	< 1	> 94.7
1.6	< 1	> 99.98	< 1	> 99.96	< 1	> 94.7

Table 2

*Reduction of viable cell count in onion flakes by gamma radiation*

(The cell count of coliform bacteria was below the 1/g level even in the untreated samples)

Radiation dose (Mrad)	Total viable aerobic cell count/g	Reduction of total viable cell count (%)	Mould count/g	Reduction of mould count (%)
0	260	—	110	—
0.1	70	73.1	9	91.8
0.2	15	94.2	3	97.3
0.8	< 1	> 99.6	< 1	> 99.1
1.6	< 1	> 99.6	< 1	> 99.1

Table 3

*Turbidity in the filtrate of the respective suspensions of untreated and radiation treated powdered onion, after incubation at 30°C for 24 hours*

(The test was carried out three weeks after irradiation of the onion powder)

Radiation dose (Mrad)	Optical density at 650 nm
0	0.67
0.1	0.32
0.2	0.16
0.8	0.02
1.6	0.03



The COD values of the untreated and treated samples are given in Table 4 in ppm saccharose equivalent.

Comparative ranking of the smell of untreated and radiation treated powdered onion samples was carried out on the day following irradiation and after storage for 6 weeks at ambient temperature. The smell of onion flakes

Table 4

*Results of the chemical oxygen demand (COD) tests*

(The data in the table are average values of two parallel tests)

Dried product	Radiation dose (Mrad)	COD value (saccharose ppm)
Powdered onion	0	760
	0.1	910
	0.2	900
	0.8	950
	1.6	1 040
Onion flakes	0	760
	0.1	420
	0.2	550
	0.8	460
	1.6	580

was tested only after 6 weeks storage. Ranking was carried out in the order of the intensity of the characteristic onion smell. Rank sums as a function of radiation dose are illustrated in Figs 1 and 2.

The sensory ranking of colour was based on the lightness of the colour of the dried onion. (The lowest value was given the sample of lightest colour.) The results of colour testing upon irradiation are shown in Fig. 3, those of the tests carried out after six weeks storage in Fig. 4.

#### 4. Conclusions

Investigation of the viable cell counts (Tables 1 and 2) show that to achieve a reduction to one tenth treatment with 0.1—0.2 Mrad is required and a reduction of two orders of magnitude, satisfactory from the practical point of view, requires 0.5—0.6 Mrad. Because of the low count of coliform bacteria in our samples before irradiation, its reduction could not be determined reliably. However, according to data of relevant literature (THORNLEY, 1963; MOSSEL & DE GROOT, 1965; MOSSEL, 1966; MOSSEL *et al.*, 1968) the

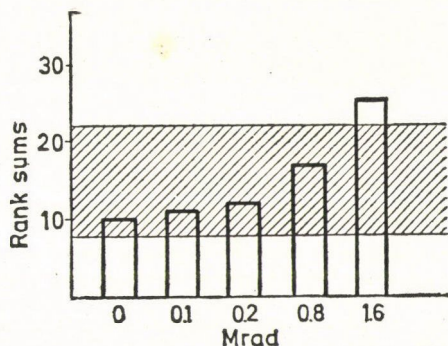


Fig. 1. Results of ranking tests of the smell of onion powder the day after irradiation. The panel consisted of 5 members. (Increasing values indicate increasing losses in characteristic onion smell.) The shaded zone marks the region of rank sums not differing significantly ( $P \leq 0.05$ ) (KRAMER, 1960)

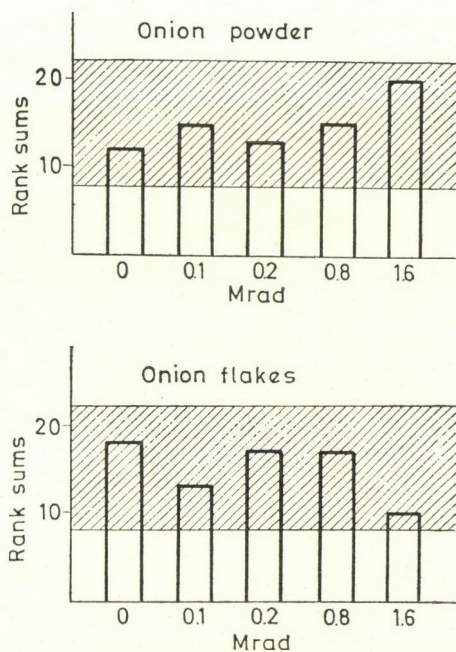


Fig. 2. Results of ranking tests of the smell of onion powder and flakes 6 weeks after irradiation. The panel consisted of 5 members. (Increasing values indicate increasing losses in characteristic onion smell.) The shaded zone marks the region of rank sums not differing significantly ( $P \leq 0.05$ ) (KRAMER, 1960)



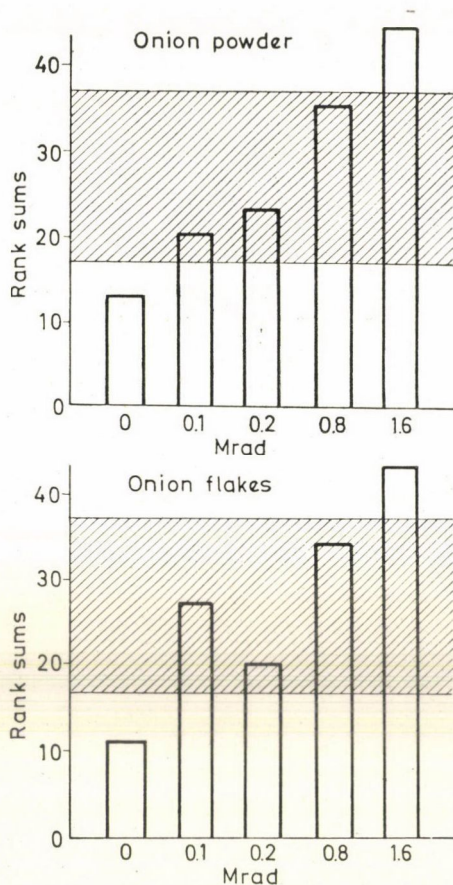


Fig. 3. Results of ranking tests of the colour of the onion powder and flakes the day after irradiation. The panel consisted of 9 members. Panelists ranked the coded samples in order of lightness, placing that of the lightest colour in the first place and the darkest one in the last place. The shaded zone marks the region of rank sums not differing ( $P \leq 0.05$ ) significantly (KRAMER, 1960)

radiation sensitivity of bacteria belonging to the family of *Enterobacteriaceae* is high even in products of low water activity and 0.6 Mrad is sufficient for their destruction in an environment of the same water activity as that prevailing in dried products ( $a_w$  0.35–0.5). The cell count of survivors was found to be below  $10^{-2}/g$ .

Ionizing radiation also affects other quality characteristics of dried onions. The intensity of the characteristic onion smell diminishes and an off-odour, resembling that of caramel appears (Fig. 1) and a brownish tint increases with increasing radiation dose (Fig. 3). However these symptoms become practically important only at doses above 0.8 Mrad which exceeds the treat-

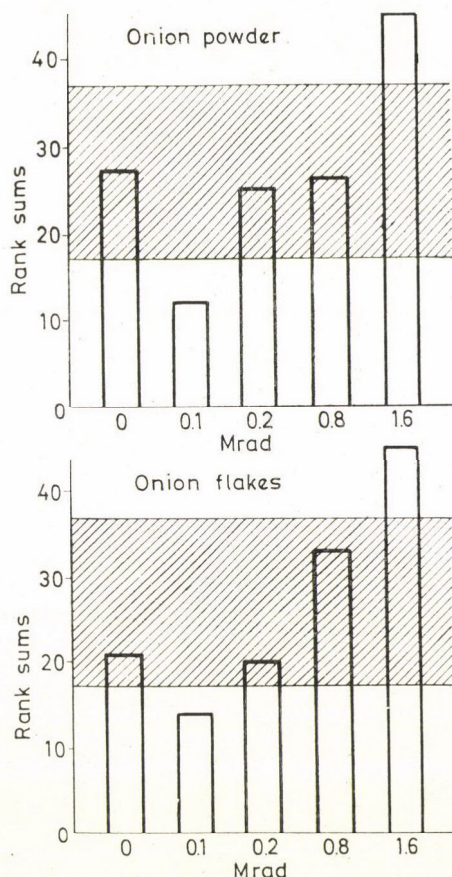


Fig. 4. Results of ranking tests of the colour of onion powder and flakes 6 weeks after irradiation. The panel consisted of 9 members. Panelists ranked the coded samples in order of lightness, placing the sample of lightest colour in the first place and the darkest one in the last place. The shaded zone marks the region of rank sums not differing ( $P \leq 0.05$ ) significantly (KRAMER, 1960)

ment necessary to reduce cell count. Apart from this the differences observed in untreated and radiation treated samples immediately upon irradiation are eliminated (off-odour) or diminish (colour) during storage following treatment, if the samples are packed in Cellothene pouches (Figs. 2 and 4). These results are in good agreement with those obtained when dried onions of Hungarian manufacture were investigated (FARKAS *et al.*, 1970) and with the findings of other researchers (SCHROEDER, 1961; N. I. R. R. H., 1971).

The COD values of the dried samples obtained with the bichromate method (SAGUY *et al.*, 1970) were not affected by irradiation above the level of fluctuation inherent in the method (Table 4). Comparing this experience



with the sensory evaluations (Fig. 1) it may be concluded that the COD value is not suitable for the characterization of the intensity of the smell of dried onions or the extent of off-odour as a consequence of irradiation.

Certain authors maintain that irradiation in the dose range required to reduce cell count has a tenderizing effect upon dried onion and thus the time requirement of rehydration or cooking is reduced. According to SCHROEDER (1967) dried onion requiring 10 min cooking, after treatment with 0.4 Mrad becomes sufficiently soft after cooking for 1 min. Bulgarian experiments have shown the cooking time requirement of dried onions to decrease from the initial 30–40 minutes to 25 minutes after treatment with 0.5 Mrad, to 15 minutes with 0.8 Mrad and to 10 minutes when treated with 1 Mrad. Treatment between 0.5 and 1.0 Mrad increased the water absorption of the samples as well by a few per cent, however above 1.5 Mrad the water absorption of the irradiated sample was lower than that of the untreated one (N. I. R. R. H., 1971). With respect to tenderization, of the dried vegetables, onions seem to be the most sensitive (SCHROEDER, 1961; 1962; 1967; DE ZEEUW & VAN KOOY, 1968; KÁLMÁN & FARKAS, 1970; FARKAS *et al.*, 1970). According to SCHROEDER (1967) the optimum radiation dose for the tenderization of onion flakes is 0.3 Mrad and in his experience the shorter cooking time needed in the case of the radiation treated onion flakes resulted in a product much richer in aromatic substances than the control sample.

A fraction of the radiation doses (0.05 Mrad) required for reducing cell count and tenderization is sufficient to destroy insects accidentally present in dried onions, in every stage of their development (TÖRÖK & FARKAS, 1960; FARKAS, 1966).

In the American experimental irradiation of dried products (SCHROEDER, 1967) the acceptability and wholesomeness of the irradiated products was also tested in animal feeding experiments and the results proved the irradiated products to be wholesome. The costs of cell count reduction by irradiation, on the basis of other publications (SCHROEDER, 1967; BDSA, 1968; BALÁZS-SPRINCZ, 1969) may be estimated to be about 0.5–1.0 Ft/kg.

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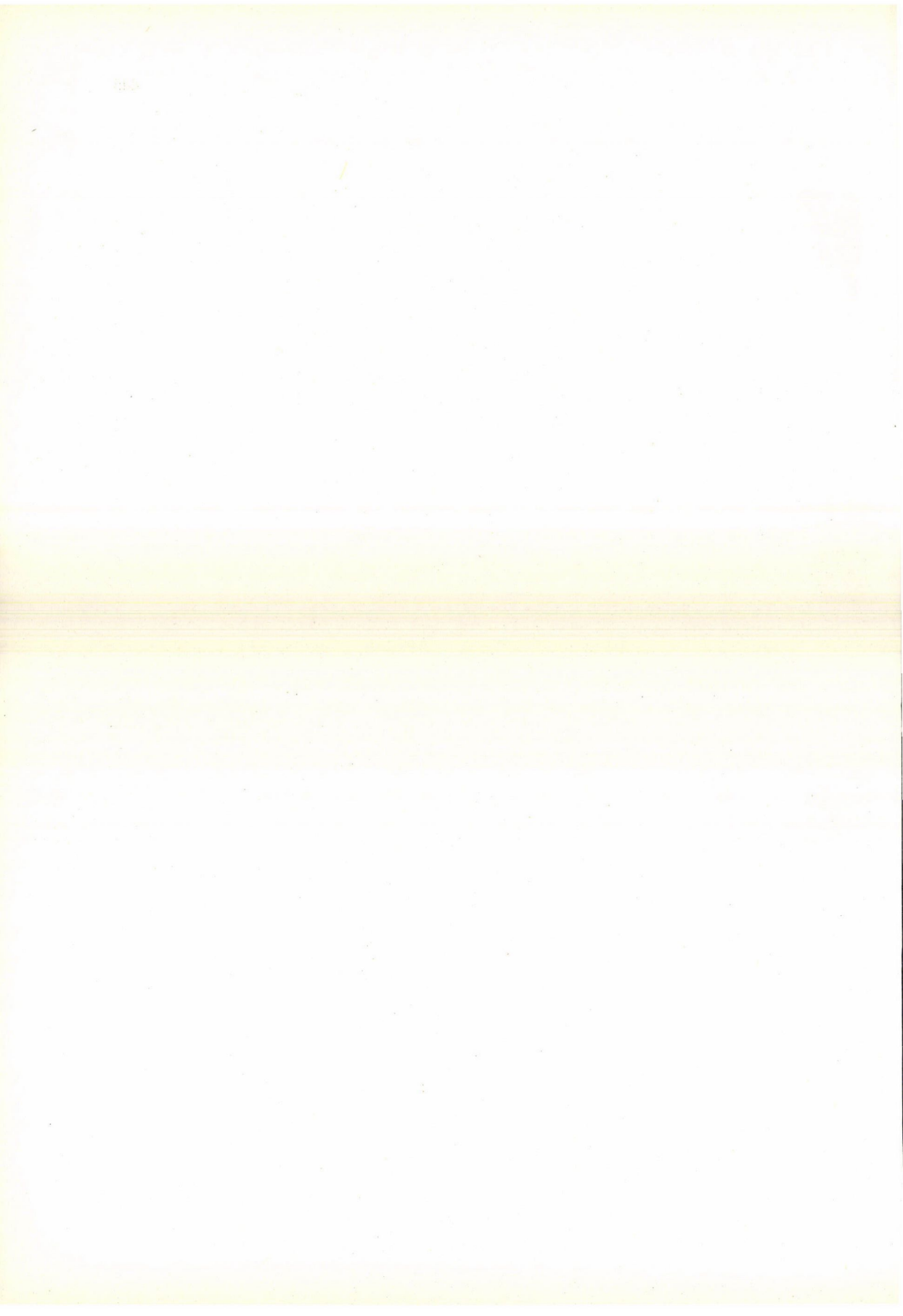


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## THE ROLE OF NITROGEN AND PHOSPHORUS IN THE PROTEIN SYNTHESIS OF *CANDIDA* *GUILLIERMONDII* CULTIVATED ON N-ALKANES

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*Candida guilliermondii* as test organism was propagated in synthetic medium where 1% of n-alkanes ( $C_{14}$ — $C_{18}$ ) served as C-source and 0.5%  $(NH_4)_2SO_4$  as N-source,  $KH_2PO_4$  was used as a P-source in quantities providing 0.1—0.005% P in the medium. The medium contained all other minerals necessary for growth.

Batch fermentation was carried out in fermentor tubes.

From the experiments it was concluded that in a culture medium containing n-alkanes, the necessary nitrogen concentration in the culture medium is 0.1%, (0.5% ammonium sulfate), utilization is 60—70%. The necessary phosphorus content is 0.025%, or the quantity of superphosphate used in practice is 0.34% and utilization is 60%. The experiments have indicated that the most favourable ratio of nitrogen to phosphorus nutrients added to the culture medium is 4 : 1. To increase the nitrogen and phosphorus concentration of the culture medium is not advisable because it does not enhance the growth capacity of yeast, nor is there any significant increase in yield.

The production of protein from hydrocarbons is being studied in a number of countries. The technology of cultivating yeast on Diesel oil or normal alkanes has reached the stage of, or is on the way to, industrial realization. Research work concerned with the microbiological utilization of hydrocarbons, the production of biomass of high protein content, has been going on in this country for several years. This research work comprises the selection of raw materials, the study of the properties of microorganisms (yeasts, bacteria) that can be used for protein production and the development of production technology. We have achieved noteworthy results in the development of the production technology of yeasts on normal alkanes on laboratory and pilot plant scale. Research work is carried on in order to reduce the production costs of biomass with high protein content by improving production technology for a better utilization of normal alkane and nutrient salts.

When yeast is cultivated on normal alkanes, nitrogen and phosphorus containing salts play an important role in protein synthesis. Of the nutrients, it is the nitrogen and phosphorus that must be added in the largest quantity to the synthetic culture medium, consequently their optimal utilization is also a question of economy. The growth of *Candida guilliermondii* on normal alkane, and the composition of the yeasts formed were studied, as a function of the quality and quantity of the nitrogen source. In addition, the effects of the



nitrogen : phosphorus ratio was studied, taking into account growth and composition of the yeast.

An account on the effect and role of the nitrogen source had been given earlier (SIMEK *et al.*, 1967, 1969a, b, c). In these experiments the nitrogen content of the culture medium was set by adding 0.5 or 1.0% ammonium sulfate and 0.25 or 0.5% urea, respectively, to ensure the equivalent nitrogen level. It was found that increasing the ammonium sulfate concentration had no significant effect on the generation time, nor on the composition of yeast. If nitrogen requirement is ensured with 0.25% urea no change occurs in the generation time, but the protein content of yeast increases significantly. The increase of the urea concentration reduces the generation time of yeast, and causes a further increase of protein. Experiments were also carried out by using as nitrogen source ammonium hydroxide simultaneously with ammonium sulfate. 70% of the required nitrogen was provided by ammonium hydroxide. The results obtained were similar to the values obtained in experiments with ammonium sulfate only. According to rentability studies, taking into account the quality of the yeast produced, and the generation time of yeast, a mixture of ammonium hydroxide and  $(\text{NH}_4)_2\text{SO}_4$  may be considered as the nitrogen source for yeast production on normal alkanes. Based on the results briefly described in the foregoing, experiments were carried out to study the effects of phosphorus concentration of the culture medium, to establish the ratio of the nitrogen and phosphorus nutrients, taking into account generation time, yield and composition of the yeast *Candida guilliermondii* yeast to be propagated. For this purpose few literary data are available on the phosphorus requirement of microorganisms, or to be more exact, of yeasts propagated on n-alkanes (ARIMA *et al.*, 1965; FRITSCH, 1968; FUJITANI, 1965, 1966; NORDHEIM *et al.*, 1967; SHUKLA & DUTTA, 1967; TELEGINA, 1967). The available data are not unambiguous either, thus the following experiments were carried out to clarify this problem from our point-of-view.

## 1. Materials and methods

### 1.1. Composition of the culture medium used

The test organism used was *Candida guilliermondii* originating from the stock collection of our Institute.

A synthetic medium was used to propagate the yeast. Potassium chloride, magnesium sulfate, zinc sulfate, manganese sulfate and ferric sulfate were added to the culture medium, prepared with tap water. The nitrogen source was ammonium sulfate, and the phosphorus requirement was provided by potassium dihydrogen phosphate.

In view of the results of the preliminary experiments described above,



0.5% ammonium sulfate was used in the culture medium and this nitrogen level, which corresponds to 0.1% was not modified during the experiments. By contrast, the phosphorus concentration was varied over a range of 0.1 to 0.005%, which corresponds to a potassium dihydrogen phosphate concentration ranging from 0.44 to 0.022%. Thus the nitrogen : phosphorus ratio in the culture medium was studied between 1 and 20. The carbon source used was a mixture of  $C_{14}$ — $C_{18}$  normal alkanes, in a concentration of 10 g/litre.

### 1.2. Preparation of the inoculum

In these experiments the yeast *Candida guilliermondii*, maintained on a solid malt culture medium was propagated. From the solid culture medium the yeast was transferred into a liquid culture medium containing 5% malt, and after multiplication into a culture medium containing 5% molasses. Yeast growth was promoted in this medium by incubation in a shaker. The next step was propagation under intensive aeration in a laboratory fermentor in a medium containing 5% molasses. When propagation was completed the yeast was separated from the medium by means of a centrifuge, the yeast thus obtained was washed twice, then, for adaptation, propagated under intensive aeration, in a culture medium containing normal alkanes, in laboratory tube fermentors. After completion of propagation the adapted yeast was separated from the medium by centrifugation, and washed with water. The thick yeast creme obtained was then used as inoculum in further experiments. 20—25% inoculum was used in the experiments as related to normal alkanes.

### 1.3. Cultivation of the yeast *Candida guilliermondii* on normal alkanes

For the propagation experiments laboratory scale glass fermentors, so-called tube fermentors, of 400 ml capacity equipped with a Gl sintered glass filter were used. Batch propagation technology was applied. Rate of aeration was 200 m<sup>3</sup> air per m<sup>2</sup> fermentor ground area, i.e. 1 000 litre air/hour/litre culture medium. The temperature of the nutrient medium was kept at 30—32°C, the pH was set between 4.5 and 5. Since during fermentation acid was formed, sodium hydroxide was added to maintain pH at a constant level. In previous experiments ammonium hydroxide had been used for this purpose, however, in these experiments this would have upset the adjusted nitrogen : phosphorus ratio. Fermentation in these experiments was carried on for 8 hours.

### 1.4. Analysis of the yeast

After completing the propagation of yeast, the yeasts were separated by centrifugation, then washed twice by suspension in water and subsequent centrifuging. The yeast mass was dried at 60°C with an infrared lamp. Nitrogen



and the raw protein were determined by Kjeldahl's method. To determine the phosphorus content the dried yeast was incinerated, dissolved in acid, precipitated with magnesia mixture, dissolved again and titrated with an ethylene diamine tetra-acetic acid-sodium solution. The disturbing effect of  $\text{Fe}^{+3}$ -ions was eliminated by adding ascorbic acid prior to titration.

The generation time of yeast was calculated on the basis of the solids content of the inoculum and of the yeast produced during 8 hours. When calculating the generation time the lag phase was included in time  $t$ , therefore, the generation time thus established was called virtual generation time.

## 2. Results

With each nitrogen-phosphorus ratio studied 10 parallel fermentations were carried out.

The results are summed up in Figs 1, 2, 3 and 4 and in Table 1.

The nitrogen : phosphorus ratios applied were 1, 2, 4, 9 and 19, respectively. During each fermentation the phosphorus concentration was reduced to about half of the initial value, while the nitrogen level was maintained at a constant level.

As seen in Fig. 1, there is a significant difference in the virtual generation time and yield of nitrogen : phosphorus ratios 9 and 19. This means that the reduction of the phosphorus content of the culture medium extends the generation time of yeast, therefore, since in these experiments the propagation time was fixed in 8 hours, the yield was lower than in experiments with a shorter generation time.

Fig. 2 shows the ash, phosphorus and nitrogen contents of the dry yeast in per cent. There is a significant difference in both ash and phosphorus contents at nitrogen : phosphorus ratios 9 and 19, respectively. A significant difference in nitrogen, i.e. raw protein content, appears only at nitrogen : phosphorus ratio 19.

On the basis of the analyses carried out the utilization of added nitrogen and phosphorus, as incorporated by the yeast cells on varying the nitrogen : phosphorus ratio, were calculated. The results are shown in Fig. 3.

The figure shows clearly that the optimum nitrogen utilization is about 60—70% at nitrogen : phosphorus ratios 1 to 4 and it decreases rapidly at higher ratios. Also the utilization of phosphorus shows a maximum value of about 60—70% at nitrogen : phosphorus ratio 4 and decreases significantly at higher phosphorus levels (nitrogen : phosphorus ratios 1 or 2). [A slight decrease appears also at lower phosphorus concentrations (nitrogen : phosphorus ratios 9 and 19), but these changes are not significant.] This fact indicates that when *Candida guilliermondii* is propagated on normal alkanes, the threshold value of the phosphorus content in the culture medium is rather high, even in case

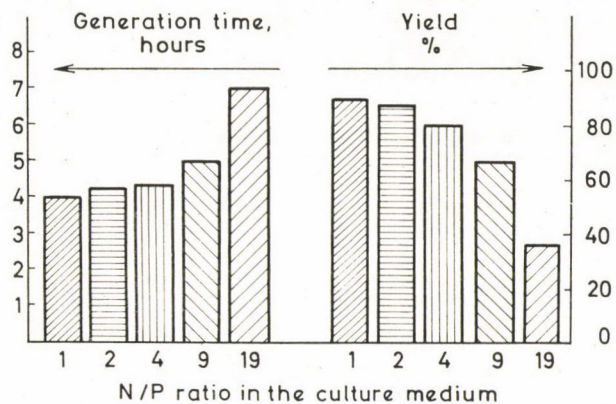


Fig. 1. Generation time and yield of yeast in culture media of varied N/P ratio

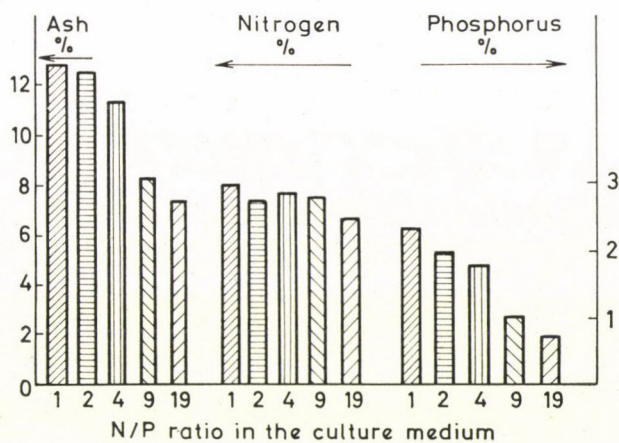


Fig. 2. Ash, nitrogen and phosphorus content of dried yeast grown in culture media of varied N/P ratio

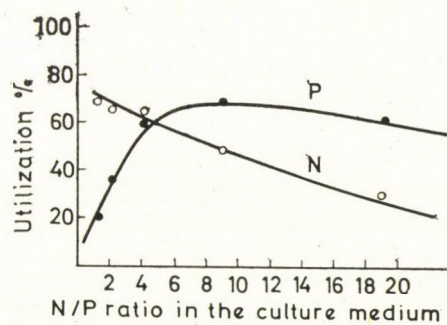


Fig. 3. Nitrogen and phosphorus utilization of yeasts propagated in culture media of varied N/P ratio



of partial phosphorus deficiency, and that only about 60% of the phosphorus available to the yeast is incorporated in the cells. Fig. 4 shows the nitrogen : phosphorus ratio in the yeast cell as a function of the nitrogen : phosphorus ratio in the medium.

With the decrease of the nitrogen : phosphorus ratio in the culture medium, the proportion of nitrogen and phosphorus in the yeast cell steadily increases, which means that the nitrogen content in the yeast produced is

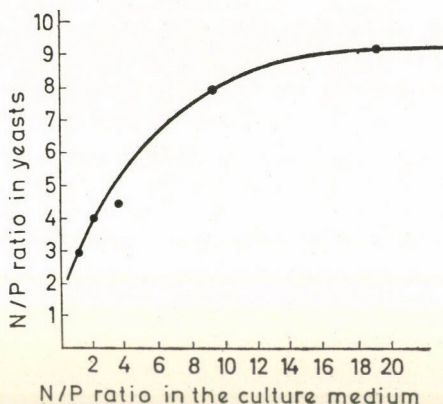


Fig. 4. N/P ratio in yeasts propagated in culture media of varied N/P ratio

practically constant, while the phosphorus content of the yeast decreases.

The results summarized in Table 1 were derived from 10 replicates each at each phosphorus concentration.

As is seen, significant change was achieved by the increase of the phosphorus content, or, in other words, by the decrease of the N/P ratio to the  $N/P = 4$  level. The state of equilibrium for generation time and for protein or N content was reached at ratio 9. As regards yield, or the per cent yeast solids as related to the added n-alkanes, changes are very highly significant up to ratio 4. By further reduction of the ratio some increase of yield may be achieved, however, this is too small to be significant at the given number of samples.

### 3. Conclusions

From the data obtained at a constant N-level in the substrate the change of added N and P utilization, or incorporation into the cell, as a function of the changing N/P ratio was derived. The optimum N utilization is around 80% and this value is in good agreement with that obtained in earlier experiments in which the effect of N added in the form of  $(NH_4)_2SO_4$  was investigated.

This value was obtained at an N/P ratio of 1. The maximum of phosphorus utilization was found to be about 60–70%. This value was obtained at N/P=4. At a higher phosphorus concentration utilization decreases to a significant extent (at  $P = 0.05$  level). There is a slight decrease in utilization at concentrations below this value, however, these changes are not significant. This fact permits of the conclusion that the threshold value of phosphorus is fairly

Table 1

*Growth and yield of yeast at different N/P ratios. Mean of 10 parallel samples*

N/P in the medium (No. of steps)		Generation time	Yield %	Ash %	P %	N %
				in the yeast		
1	mean	4.0	89	12.9	2.32	8.00
	standard deviation	0.84	13.9	1.72	0.393	0.672
2	mean	4.1	80	12.6	2.00	7.76
	standard deviation	1.04	17.6	1.35	0.398	0.709
4	mean	4.2	80	11.6	1.79	7.87
	standard deviation	0.83	14.5	0.43	0.344	0.458
9	mean	4.9*	60**	8.2***	0.986	7.79
	standard deviation	1.32	14.5	0.99	0.3833	0.520
19	mean	7.07***	36***	7.31	0.765	7.03**
	standard deviation	1.59	14.1	1.13	0.4240	0.544

The significances of the differences are established from the standard deviations of the mean values of two subsequent steps

\* significant ( $P \leq 0.05$ ),

\*\* highly significant ( $P \leq 0.01$ ),

\*\*\* very highly significant ( $P \leq 0.001$ ).

high, when *Candida guilliermondii* is cultivated on a substrate of n-alkanes and even in case of partial phosphorus deficiency only about 60% of the available phosphorus is incorporated in the cell. With decreasing N/P ratio in the medium the nitrogen and phosphorus utilization in the cell steadily increases.

On the basis of our observations it was established that in a substrate containing 1% of n-alkanes, the optimum N/P ratio is 4 : 1, if the N-source essential to obtain the theoretically possible yeast yield, in the given case *Candida guilliermondii* yield, is 0.5%  $(\text{NH}_4)_2\text{SO}_4$ . The addition of more phosphorus is unnecessary, since it is not adequately utilized and does not yield significant improvement neither in the growth nor in the composition of the yeast.



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PHYSICAL AND CHEMICAL CHANGES IN IRRADIATED  
SALMON (*ELEUTHERONESMA TETRADACTYLUM*)  
AND BLACK POMFRET (*PARASTROMATEUS NIGER*)

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Dark muscles from both Indian salmon and black pomfret contained more heme pigments and lipids than the corresponding white muscle and are prone to radiation-induced oxidative changes as assessed in terms of formation of free fatty acids and TBA reactive substances.

Differences in the muscle tissues were noticed in the form of production of off-odours by irradiation. The dark muscles showed typical rancid odours above 1.0 Mrad, whereas the white muscles exhibited burnt type of odours.

Studies on the absorption spectra of heme pigments indicated that oxidation of oxymyoglobin to metmyoglobin takes place at 0.5 Mrad under aerobic conditions. Anaerobic atmosphere during irradiation favoured the regeneration of oxymyoglobin.

Radiation dose of 0.5 Mrad brings about loss in extractability of fibrillar proteins without causing any alteration in the solubility of sarcoplasmic proteins.

Compositional factors play an important role in determining the amenability of fish varieties to different processing treatments. Studies on radiation dose responses provide basic information required for selection of tolerance dose and other processing parameters. Exudation of drip in Bombay duck either by freezing or irradiation can be ascribed to the susceptibility of fibrillar proteins to denaturation (GORE & KUMTA, 1970; KUMTA & GORE, 1970), while blackening in shrimps due to enzymes of melanosis leads to limited acceptability of ice stored or irradiated shrimps (KUMTA *et al.*, 1970; NOVAK & LIUZZO, 1965). White pomfret, a medium fatty fish, undergoes oxidative changes leading to yellow discolouration of skin during frozen storage or on irradiation (KAMAT & KUMTA, 1972). These changes in Bombay duck, shrimps and white pomfrets can be minimised by dip treatments in NaCl, NaTPP (GORE & KUMTA, 1970), blanching (KUMTA *et al.*, 1970) and vacuum packaging (KAMAT & KUMTA, 1972), respectively.

Indian salmon and black pomfret belong to medium fatty fish varieties in which the dark muscle tissues, because of the proximity of lipids and heme proteins, may be prone to loss of natural colour and development of oxidative rancidity. The purpose of this paper is to investigate radiation dose responses of white and dark muscle tissues of Indian salmon and black pomfret in terms of solubility changes of textural proteins, oxidation of lipid components and changes in hemoproteins. Effects of anoxic conditions for the suppression of oxidative changes have also been studied.



## 1. Materials and methods

### 1.1. Preparation of sample

Fresh Indian salmon and black pomfret available in local market were brought to laboratory in ice. They were cleaned with water before and after evisceration. A cut was made along the lateral lines of the fish. The skin was separated by running the knife in between the skin and muscle. The dark muscle was then separated from the white muscle. The separated muscles were kept in ice until further use.

### 1.2. Chemical composition

The chemical composition was determined with three different lots of samples. The average values of these samples are given in text. Dark and white muscles were assayed separately for their composition.

*1.2.1. Moisture.* Samples (5 g each) were dried at 105°C in an oven till constant weights were obtained and the percentage was determined.

*1.2.2. Proteins.* The sarcoplasmic proteins of minced muscles were extracted with 0.05 *M* phosphate buffer, pH 7.5 and myofibrillar proteins by the same buffer containing 5% NaCl according to the method of KING (1966). Protein nitrogen was determined by nesslerization according to the method of UMBREIT and his co-workers (1957).

*1.2.3. Lipids.* Chloroform and methanol in the ratio of 1 : 2 was used for the extraction of tissue lipids (BLIGH & DYER, 1959) and free fatty acids (DUNCOMBE, 1963), triglycerides (VANHANDEL & ZILVERSMIT, 1957) and lipid phosphorous (FISKE & SUBBAROW, 1925) were estimated.

*1.2.4. Heme pigments.* 10 and 20% of the dark and white muscle respectively were homogenized in chilled distilled water. The homogenate was centrifuged at 10 000 G for 20 min at 0–2°C. 2 ml aliquots were placed on the top of DEAE-cellulose column equilibrated with Tris buffer, 0.05 *M*, pH 8.6. Myoglobin was eluted with the same buffer, retaining hemoglobin on the column. The optical density of the pooled myoglobin fraction was noted at 408 nm. The absolute concentration of myoglobin was obtained by making use of extinction coefficient  $1.68 \times 10^5$  for sperm whale myoglobin.

### 1.3. Dip treatment

Minced white muscle (5 g) was tied in a muslin cloth and dipped in Na-tripolyphosphate (NaTPP) solution for 40 sec. The samples were then removed and the excess NaTPP solution was allowed to drain away by holding over a funnel for 15 min. Free salt solution adhering to the muscle tissue was removed by gently pressing with tissue paper.

#### 1.4. Packaging

Dark and white muscle were packed in polycell bags (polyethylene 1.5 mil/cellophane 400 MST laminate) under vacuum after repeated flushing and evacuation with nitrogen gas. Since the packaging material used was permeable to oxygen, each bag was further sealed under vacuum in a metal can to maintain strict anaerobic conditions during irradiation.

#### 1.5. Irradiation

Irradiation was done at 0.5—2.0 Mrad at a dose rate of 0.85 Mrad/hr in a Gamma cell -220,  $^{60}\text{Co}$  source (22 500 curie) at 0—2°C.

#### 1.6. Chemical evaluation of oxidative changes

The fish muscles were examined for oxidative changes in terms of peroxide number, 2-thiobarbituric acid (TBA) value and free fatty acid (FFA values) according to WAGNER and his co-workers (1947), WITTE and his co-workers (1970) and DUNCOMBE (1963). Oxidative changes in heme pigments extracted in water were determined by recording the absorption spectra in the visible region (300—700 nm) in a Perkin—Elmer automatic recording spectrophotometer. The assessments of oxidative changes were done within fifteen minutes after irradiation.

### 2. Results and conclusions

Colour, flavour and texture form important attributes which determine the freshness and acceptability of fishery and other meat products. Selection of tolerance dose either for pasteurisation or sterilisation will be governed by the degree of changes in the tissue constituents. Sensory evaluation of Indian salmon and black pomfret fillets exposed to different doses indicated that doses up to 0.3 Mrad would be more suitable for radurisation since at these doses no detectable changes occur in the original freshness of these fish varieties. Radiation dose responses studied in terms of oxidative changes, discolouration and loss in texture reveal that these fish varieties are not amenable to radapertisation at 4.5—5.6 Mrad; unless combination treatments comprising vacuum packaging, blanching and cryogenic temperature during irradiation are adopted as with ham, bacon, chicken and beef (COLEBY *et al.*, 1961; HEILIGMAN *et al.*, 1967; WADSWORTH & SHULTS, 1966).

#### 2.1. Chemical composition

The data on the chemical composition of Indian salmon and black pomfret muscle tissues are given in Table 1



Table 1

*Biochemical composition*

Separation of dark and white muscles and estimation of moisture, proteins (KING, 1966),  
rus (FISKE & SUBBAROW, 1925), and free fatty acids

Fish	Type of muscle	Moisture %	Total	Proteins %	
				Fibrillar	Sarcoplasmic
Indian salmon	*Dark	66.0	16.0	12.0	4.4
	White	71.0	18.8	14.0	4.8
Black pomfret	Dark	60.61	17.0	12.5	4.5
	White	62.0	22.0	17.2	5.0

Mb=myoglobin; Hb=hemoglobin; TG=triglycerides; PL=phospholipids; FFA=free fatty acids.  
The range of maximum and minimum value is shown in parantheses.

\* In both fishes dark muscle constitutes about 12–15% of the entire muscle

It can be seen from the results that dark muscle tissues contain more lipids and heme pigments than the corresponding white muscle tissues, myoglobin *per se* accounting for 80–85% of total heme pigments. Of the total lipids, 70–80% is constituted by triglycerides and 8–16% by phospholipids. Such compositional differences have been reported for cod (BLIGH & SCOTT, 1966), Baltic herring (BOSUND & GANROT, 1969) and tuna fish varieties (BROWN, 1962).

## 2.2. Radiation response of dark and white muscle tissues

Compositional differences between dark and white muscle tissues are reflected in their differences in type of radiation induced odours and qualitative and quantitative changes in myoglobin. These results are shown in Table 2.

The rancid type of odour at doses above 0.5 Mrad were conspicuously seen with dark muscle tissue while "burnt" type of odours were characteristic of irradiated white muscle tissues. The precursors for rancid type of odours have been suggested to be the unsaturated moieties of lipids (BATZER *et al.*, 1957; DUBRAVICIC & NAWAR, 1969; MERRITT, 1966; MONTY *et al.*, 1961), while burnt type of odours have been attributed to formation of volatile sulfur compounds arising from cysteine, methionine (KOPOLDOVA *et al.*, 1958; SHIMAZU

*of muscle tissues*

heme pigments (BROWN, 1962), triglycerides (VANHANDEL & ZILVERSMIT, 1957), lipid phospho- (DUNCOMBE, 1963) were done as described in text

Heme pigments %			Lipids %			
Total	Mb	Hb	Total	TG	PL	FFA
1.2	1.0	0.2	3.2 (2.4—4.0)	2.24	0.64	0.32
0.3	0.2	0.1	0.95 (0.7—1.2)	0.66	0.19	0.06
2.2	1.6	0.6	7.0 (5.0—9.0)	5.6	0.92	0.50
0.9	0.6	0.3	3.9 (2.4—5.4)	2.93	0.71	0.26

*et al.*, 1964; MERRITT, 1966) and other soluble proteins (HEDIN *et al.*, 1960, 1961).

Figs. 1 and 2 show the oxidative changes in irradiated muscles of Indian salmon and black pomfret, respectively. The initial values of TBA, FFA and peroxide are higher in the dark muscles. On irradiation the values of FFA and TBA increased in the dark muscles. These increases were accompanied by formation of rancid odours. Formation of FFA and TBA were relatively less in the white muscle tissues. It is also evident that irradiation under vacuum suppressed oxidative changes.

The decline in the peroxide values as a result of irradiation may be possibly due to the breakdown of peroxides or their interactions with other tissue constituents.

### 2.3. Changes in colour

Fresh muscle tissue owes its colour characteristics to oxymyoglobin. Under oxidising atmosphere this pigment is converted to metmyoglobin accompanied with brown discolouration as reported for canned or frozen fishery products (AMANO, 1965; BITO, 1965).

Figs. 3 and 4 depict the spectral alterations of heme proteins from irradiated (0.5 Mrad) dark muscles of Indian salmon and black pomfret, respectively. The peaks due to oxymyoglobin are lost as a result of formation of metmyoglobin. Irradiation under anaerobic atmosphere suppressed the oxidative changes which favours the formation of oxymyoglobin. This is evidenced



Table 2

*Organoleptic attributes of irradiated muscle tissues*

The dark and white muscles were separately irradiated in a Gamma cell 220 ( $^{60}\text{Co}$  source, 22 500 Ci AECL) at  $0-2^\circ\text{C}$ . Organoleptic evaluation was done immediately after irradiation according to MIYAUCHI *et al.* (1964)

Dose, Mrad	Muscle	Indian salmon		Black pomfret	
		Score	Sensory attributes	Score	Sensory attributes
Unirradiated	Dark	10	Fresh fish odour, cherry red colour	9	Fish oil like odour, purple colour
	White	10	Fresh fish odour and colour	10	Fishy odour, appearance of a fresh fish
0.1-0.3	Dark	10	Fresh fish odour and colour	10	Appearance of tissue as in fresh fish, no change in colour
	White	10	Fresh fish odour and colour	10	No change in sensory attributes from unirradiated samples
0.5	Dark	8	Slight changes in odour, no detectable change in colour	8	Slight rancid odour, brown colour
	White	9	Fresh fish odour and colour	9	Slight irradiation odour, no change in colour
1.0	Dark	7	Rancid odour, slight brownish discolouration	6	Rancid odours, brown colour
	White	8	Slight irradiation odour, no change in colour	8	Slight rancid odour, no change in colour
1.5	Dark	6	Rancid odour, more brown discolouration	6	Strong rancid odours
	White	7	More irradiation odour, no change in colour	7	Rancid odour, slight brown discolouration
2.0	Dark	5	Strong rancid odours, brown discolouration	4	Intense rancid odours
	White	6	Slight brown discolouration, strong irradiation odour	6	Brown discolouration, strong irradiation odour

by the formation of peaks at 575 and 545 nm in case of Indian salmon and shift in the solet peak from 409 to 415 nm and regeneration of oxymyoglobin peaks at 575 and 545 nm in case of black pomfret.

The mechanism of the transformation of metmyoglobin to oxymyoglobin have not been completely elucidated. TAPPEL (1956) has suggested that  $\text{H}_2\text{O}_2$  or  $\cdot\text{OH}$  radicals formed during radiolysis of water may reduce the metmyoglobin, while SATTERLEE and his co-workers (1972) have attributed this to the presence of hydrated electron ( $e_{\text{aq}}$ ). This radical with its ability to reduce metal ions, could possibly convert metmyoglobin to oxymyoglobin.



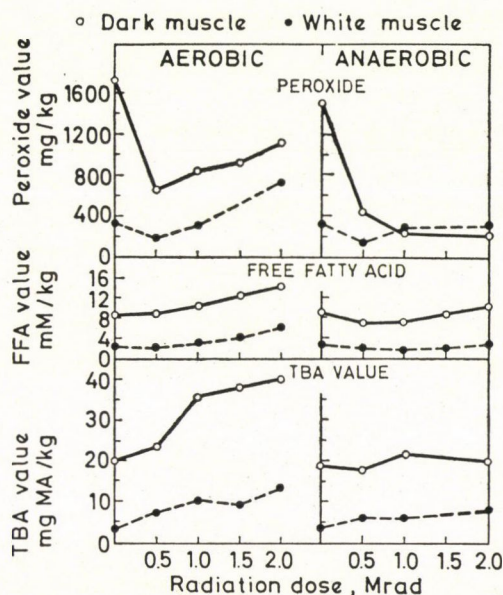


Fig. 1. Oxidative changes in irradiated Indian salmon muscle. Oxidative changes are assessed in terms of TBA (WITTE *et al.*, 1970), FFA (DUNCOMBE, 1963) and peroxide number (WAGNER *et al.*, 1947). Dark muscle tissue is more susceptible to oxidative changes than the white muscle tissue. Anaerobic packaging minimises the oxidative reactions during irradiation. [TBA values expressed as mg malonaldehyde (MA) per kg.]

#### 2.4. Loss in protein solubility

Data presented in Table 3 provide evidence to show that irradiation induces denaturation of fibrillar protein as observed in terms of loss in solubility in 5% NaCl. With increasing doses of irradiation the solubility of textural proteins steadily decreases. A pre-dip treatment in polyphosphate (10%) of the white muscle tissues prior to irradiation improved the solubility. Studies of YASUI and his co-workers (1964) on the effect of NaTPP, Na-hexameta-phosphate and pyrophosphates on the fibrillar proteins suggest that these salts greatly improve the solubility of actomyosin by dissociating it. Improvement in solubility by dip treatment prior to irradiation would suggest that actomyosin in dissociated form may not readily undergo denaturation.

Studies on dose response relationship of fibrillar proteins point out that during radappertisation the proteins may undergo irreversible denaturation. Since these proteins are associated in the maintenance of stability of texture, incorporation of NaTPP would have beneficial effect in radappertised products.



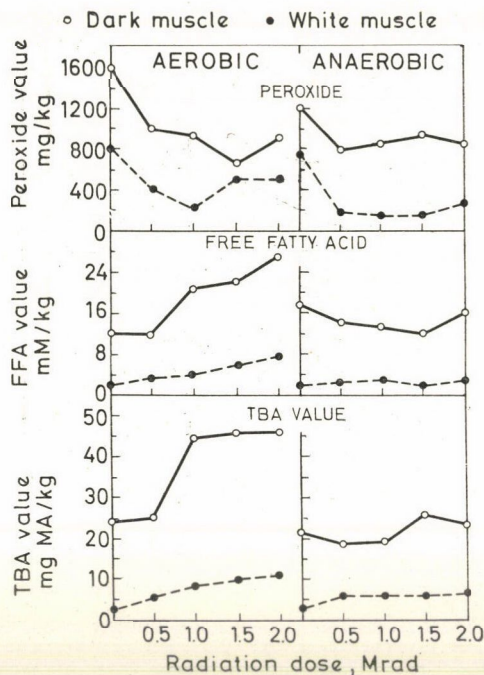


Fig. 2. Oxidative changes in irradiated black pomfret. The influence of anaerobic packaging in suppressing oxidative changes is evidenced by low values of TBA, peroxide and FFA value in irradiated black pomfret muscles

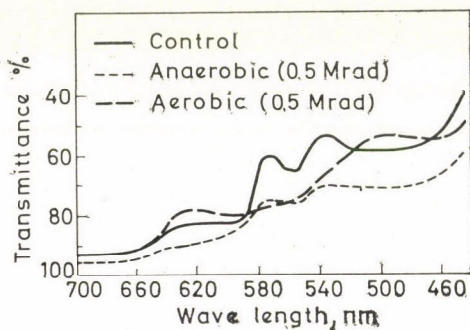


Fig. 3. Absorption spectra of heme pigments extracted from Indian salmon dark muscle. Formation of new peaks at 630 and 500 nm in the absorption spectra of heme pigments from muscles exposed to 0.5 Mrad under aerobic condition indicated oxidation of oxy-myoglobin. Irradiation under anaerobic condition retained the original spectral characteristics of oxy-myoglobin

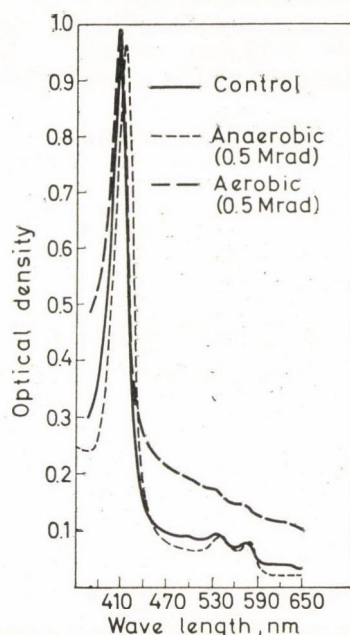


Fig. 4. Absorption spectra of heme pigments extracted from black pomfret dark muscle. Shift in the absorption maxima in the solet region from 408—415 nm indicated formation of oxymyoglobin in anaerobically irradiated muscle

Table 3

*Extractability of proteins\* from Indian salmon and black pomfret muscle tissues*

Sarcoplasmic proteins were extracted within 15 min after irradiation with 0.05 *M* phosphate buffer, pH 7.5 and myofibrillar proteins with same buffer containing 5% NaCl according to KING (1966). Protein nitrogen was determined according to UMBREIT and co-workers (1957)

Dose, Mrad	Indian salmon			Black pomfret		
	Fibrillar		Sarcoplasmic	Fibrillar		Sarcoplasmic
	No treatment	Dip in NaTPP		No treatment	Dip in NaTPP	
Unirradiated	88	88	100	82	84	100
0.5	71	85	100	75	80	98
1.0	62	75	98	71	77	98
1.5	—	—	100	60	74	100
2.0	52	61	99	59	62	100

\* Values expressed as per cent of total nitrogen.



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## RADURISATION PROCESS FOR INDIAN SALMON (*ELEUTHERONESMA TETRADACTYLUM*) AND BLACK POMFRET (*PARASTROMATEUS NIGER*)

U. S. KUMTA and V. N. MADHAVAN

(Received October 4, 1972)

A radurisation process comprising anaerobic packaging, irradiation at 0.1 Mrad and storage at 0—2°C has been described for the extension of shelf-life of Indian salmon and black pomfret. The radurised fillets showed an extension of shelf-life upto 20—25 days without much change in natural freshness characteristics as against the unirradiated controls which spoiled within 8—10 days.

Fillets exposed to higher doses of 0.2 or 0.3 Mrad and stored under aerobic conditions manifested oxidative changes in the form of rise in TBA reacting substances and formation of rancid odours. Anaerobic packaging minimised these changes.

Solubility of heme pigments decreased by 50—70% during storage in ice of fillets exposed to 0.2 or 0.3 Mrad. This effect was more pronounced during aerobic storage conditions as compared to anaerobically stored fillets.

In the preceding paper (MADHAVAN & KUMTA, 1973) it was reported that radiation doses of 0.1—0.3 Mrad did not cause any detectable change in the organoleptic qualities of Indian salmon and black pomfret fillets. The suitability of these doses for radurisation will be however determined by the extent of changes undergone by heme pigments and lipids during aerobic storage conditions of radurised fillets. Two reactions which need consideration are lipid peroxidation catalysed by heme proteins and consequent brown discolouration.

Attempts are made in this communication to assess the keeping qualities of radurised Indian salmon and black pomfret fillets on the basis of oxidative changes in lipids in terms of formation of 2-thiobarbituric acid reacting substances (TBARS) and stability of heme pigments. In addition, total bacterial counts (TBC), trimethyl amino nitrogen (TMAN), total volatile basic nitrogen (TVBN) and sensory score have been used as freshness indices. Efficiency of anaerobic packaging in minimising oxidative changes in lipids and heme pigments has also been tested.

### 1. Materials and methods

#### 1.1. Preparation of sample

Fresh Indian salmon (*Eleutheronema tetradactylum*) and black pomfret (*Parastromateus niger*) were brought from docks to laboratory in crushed ice.



The fish were washed clean in water, eviscerated, cut into fillets and washed thoroughly again.

### *1.2. Packaging*

The fillets were packed in polycell bags (Polyethylene 1.5 mil/cellophane 400 MST laminate) under vacuum after repeated evacuation with nitrogen gas. Since the packaging material used was permeable to oxygen, each bag was further sealed under vacuum in a metal can to maintain strict anaerobic conditions.

### *1.3. Irradiation*

Irradiation of the packed fillets was done at 0.1–0.3 Mrad at a dose rate of 0.85 Mrad/hr in a Gamma cell-220,  $^{60}\text{Co}$  source (Atomic Energy Canada Ltd., 22 500 Ci) at 0–2°C.

### *1.4. Storage*

Irradiated and unirradiated fillets were stored in ice in a cold room (0–2°C) till they were removed for periodical analysis.

### *1.5. Sensory evaluation*

Samples were assessed for appearance and odour using the ten point reference scale of MIYAUCHI and co-workers (1964).

### *1.6. Bacteriological analysis*

Fish homogenate (10%) was prepared aseptically in physiological saline (0.85%) and total viable count was determined in the homogenate by the standard pour plate technique using TGY agar medium containing 0.5% NaCl. The plates were incubated for 72 hrs at 30°C before counting the colonies. Results are expressed as counts/g of fish.

### *1.7. Chemical indices*

The extract of fish muscle 10% in TCA was allowed to stay overnight and filtered through Whatman No. 1 filter paper. The filtrate was used for the estimation of TVBN and TMAN. TVBN and TMAN were estimated according to the methods of FARBER and FERRO (1956) and BETHEA and HILLIG (1965), respectively. The values are expressed as mg N/100 g fish. Thio-



barbituric (TBA) values were estimated according to the method of TURNER and co-workers (1954) using 20% water extract of the tissue. The values are expressed as mg malonaldehyde (MA)/kg of fish.

### 1.8. Solubility of heme pigments

Minced muscle (5 g) was homogenised with 45 ml distilled water and was centrifuged at 10 000 g at 0–2°C. The clear supernatant was filtered through Whatman No. 1 filter paper to remove floating fat bodies. Aliquots of the filtrates were used for measuring the optical density at 408 nm in Beckman-DB spectrophotometer. Fall in O. D. was used as a measure of loss in solubility of heme pigments.

## 2. Results and conclusions

Shelf stability of fishery products is achieved by suppression of spoilage microorganisms by gamma radiation. However, the stability of tissue constituents which govern texture, flavour and colour may be adversely affected depending upon the dose (GORE & KUMTA, 1970; KUMTA & GORE, 1970; SPINELLI *et al.*, 1967; KAMAT & KUMTA, 1972; MADHAVAN & KUMTA, 1970; TAPPEL, 1956). Judicious selection of optimum tolerance dose is most essential so that it meets two requirements of the radurisation process: (i), the radurised products should exhibit extension of shelf life without major loss in freshness characteristics and (ii) the terminal spoilage pattern should be identical with that of unirradiated controls to permit olfactory rejection of the irradiated products. The latter condition is imposed based on the observations of EKLUND and POYSKY (1970) that in inoculum pack studies with *Cl. botulinum*, detection of spoilage preceding toxin development becomes possible if radiation dose and temperature of storage are suitably selected.

### 2.1. Freshness attributes of radurised Indian salmon and black pomfret fillets

Unirradiated Indian salmon and black pomfret fillets undergo rapid spoilage within 5–8 days concomitant with increase in TBC, TVBN and TMAN and fall in organoleptic score. These results are shown in Figs 1 (A & B) and 2 (A & B) which also provide information on the status of freshness indices for radurised fillets.

Radurisation at 0.1 Mrad extended the shelf-life of both the fish in fresh-like condition up to 20 days. Distinct putrid odours as in unirradiated samples were observed during the terminal storage period. At 0.2 or 0.3 Mrad doses, the organoleptic score up to 7–8 points was retained up to 25–30 days of storage. During this period there was sharp rise in TBC but TMAN and TVBN showed lag in their formation.



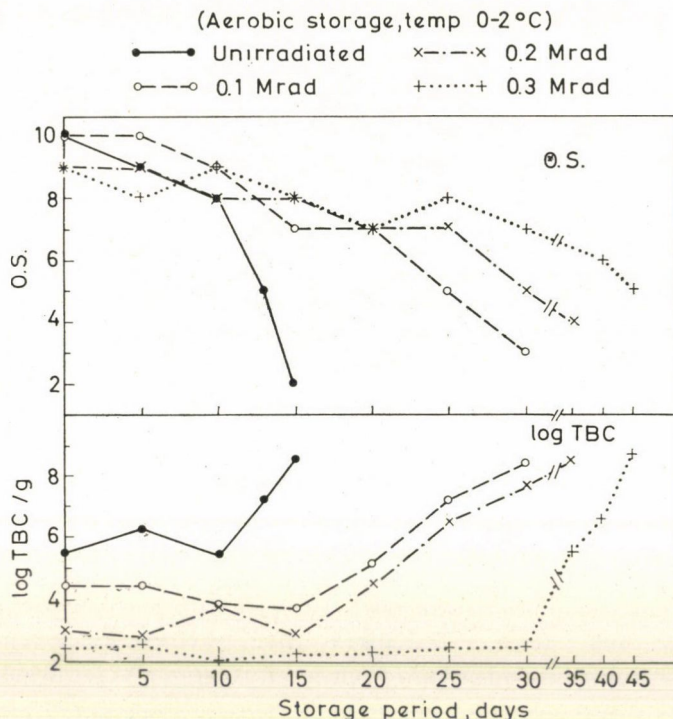
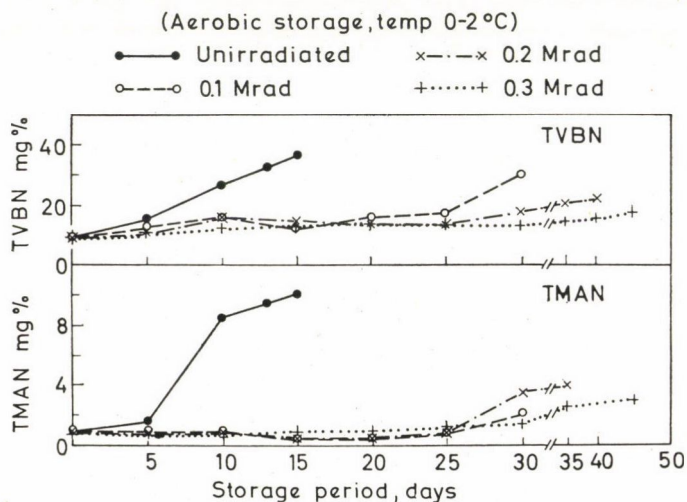


Fig. 1 (A & B). Freshness characteristics of radurised (0.1–0.3 Mrad) Indian salmon fillets. Cut fillets were exposed to 0.1–0.3 Mrad as described in the text. The radurised fillets were stored at 0–2°C and periodically assessed for organoleptic score (O. S.) (MIYAUCHI *et al.*, 1964), TBC (as in text), TMAN (BETHEA & HILLIG, 1965) and TVBN (FARBER & FERRO, 1956). Unirradiated fillets showed a rapid loss in freshness characteristics as indicated by rise in TBC, TVBN and TMAN and fall in O. S. Radurised products had better organoleptic attributes for a prolonged period



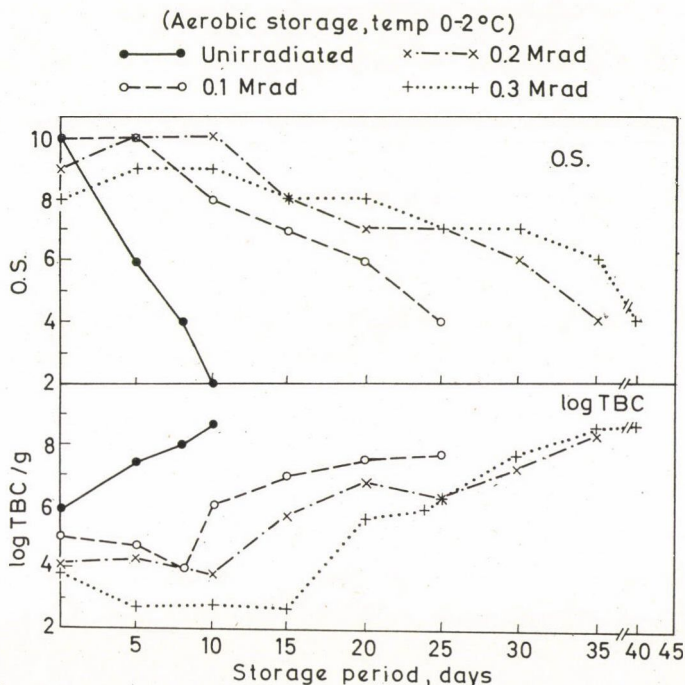
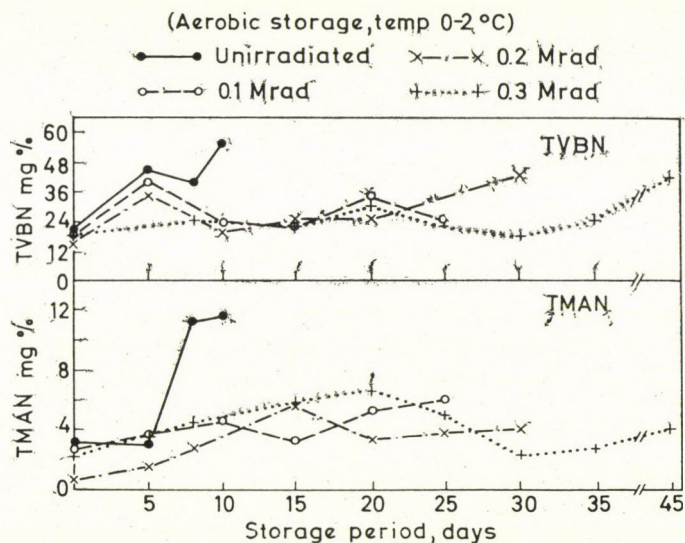


Fig. 2 (A & B). Freshness characteristics of radurised (0.1–0.3 Mrad) black pomfret fillets. Irradiation and the periodic assessment of the quality of processed samples were done by methods as described for Fig. 1. Radurised fillets retained high organoleptic attributes upto 25–30 days in contrast to rapid loss in freshness attributes in unirradiated samples





## 2.2. Oxidative changes

The cherry red colour characteristic of fresh Indian salmon and black pomfret fillets is due to the presence of reduced heme pigments (OxyHb and OxyMb). These may turn brown under aerobic storage due to oxidation. Fresh samples and samples radurised at 0.1 Mrad did not exhibit any brown discolouration till the end of the storage period. The samples exposed to 0.2 or 0.3 Mrad exhibited some fading of colour. This, however, did not affect the overall acceptability of the colour characteristics. It is interesting to note that both Indian salmon and black pomfret fillets exposed to 0.2 or 0.3 Mrad exhibited loss in heme pigment solubility (50–70%) after 30 days of storage (Fig. 3). The loss in heme pigment solubility could either be due to (i), denaturation (BARRETT *et al.*, 1965; BROWN *et al.*, 1967; CRAWFORD & FINCH, 1968) or (ii), formation of hemoprotein polymers which are rendered insoluble in the presence of lipid peroxides (DESAI & TAPPEL, 1963; NISHIDA & NISHIDA, 1965)

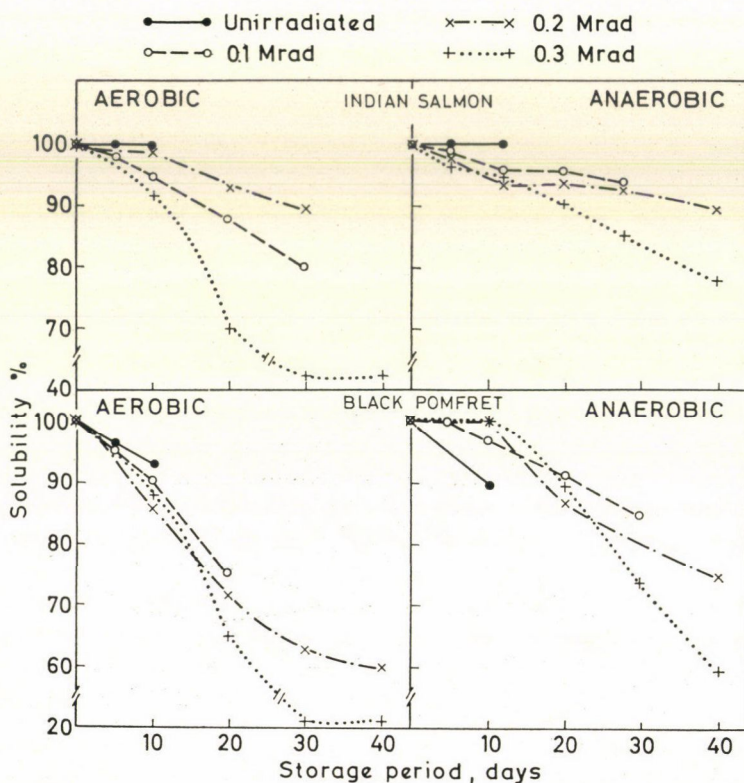


Fig. 3. Solubility of heme pigments in radurised (0.1–0.3 Mrad) Indian salmon and black pomfret fillets. The extractability of heme pigments from the fish fillets was performed as mentioned in the text. Under aerobic storage condition, the loss in solubility of heme pigments is enhanced in both Indian salmon and black pomfret fillets exposed to 0.2 or 0.3 Mrad. Samples radurised and stored under anaerobic conditions exhibited an improved heme pigments solubility pattern

Figs 4 and 5 provide data on the progressive increase in oxidative changes measured in terms of TBARS in irradiated samples stored under aerobic conditions. Correlation between development of rancid odour and formation of TBA reacting substances was also noticed in samples irradiated and stored under aerobic conditions. In samples subjected to 0.1 Mrad, spoilage odours masked the rancid type of odour, but these were conspicuous in samples exposed to 0.2 or 0.3 Mrad. It was interesting to note that initially there was gradual increase in TBA values, however, these declined after reaching a maximum peak value. This can be attributed either to interaction of TBARS with other tissue constituents such as amino acids, proteins etc. or their utilisation by the surviving microflora (SMITH & ALFORD, 1968). In contrast, vacuum packaging effectively arrested these changes.

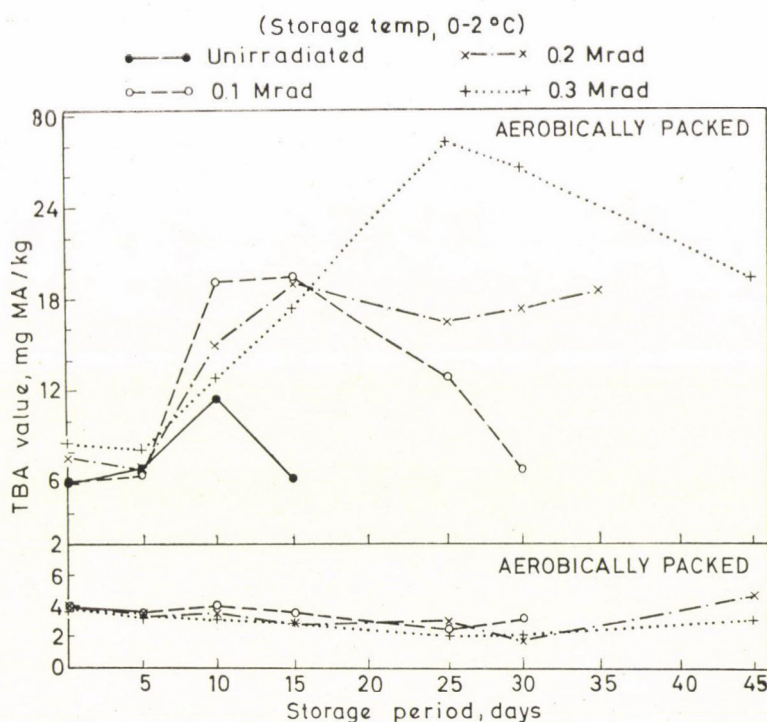


Fig. 4. Oxidative changes in radurised (0.1–0.3 Mrad) Indian salmon fillets. At intervals during storage, the samples were assessed for TBA values (TURNER *et al.*, 1954) as an index of oxidative changes. The samples exposed to 0.1–0.3 Mrad exhibited rapid rise in TBA values during storage. Anaerobic packaging effectively suppressed formation of TBA reacting substances

### 2.3. Merits of radurisation process

Indian salmon and black pomfret together with other medium fatty fish varieties constitute about 10–15% of the total catch amounting to



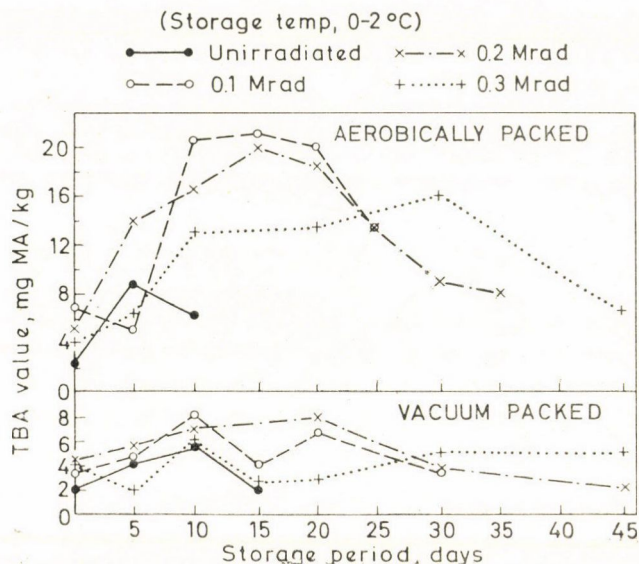


Fig. 5. Oxidative changes in radurised (0.1–0.3 Mrad) black pomfret fillets. Efficiency of vacuum packaging in the suppression of oxidative changes is evidenced by low TBA values in the samples during anaerobic storage

172 thousand tons. These fish varieties are normally not canned or frozen and the bulk of the catch is ice stored and consumed fresh. Due to the prevailing methods of handling, transportation and retailing, ice storage offers a limited storage life and hence the distribution of fish in India is restricted to the coastal belt. Extension in shelf-life by radurisation extends the inland distribution possibilities of these fishes (KUMTA & SREENIVASAN, 1970; KUMTA *et al.*, 1972).

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